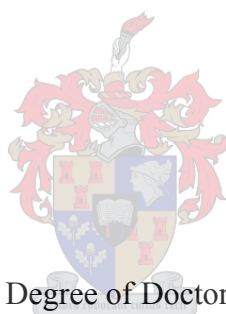


THE ISOLATION AND CHARACTERISATION OF OVINE LIVER CYTOCHROME b₅

by

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Dissertation presented for the Degree of Doctor of Philosophy (Biochemistry)

at the University of Stellenbosch.

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March 2007

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Nicolaas Lombard

Date

VERKLARING

Ek, die ondergetekende, verklaar hiermee dat die werk in hierdie werkstuk/tesis vervat, my eie oorspronklike werk is en dat ek dit nie vantevore in die geheel of gedeeltelik by enige universiteit ter verkryging van 'n graad voorgelê het nie.

Nicolaas Lombard

Datum

SUMMARY

This dissertation describes how the isolation and characterisation of ovine liver cytochrome b₅ was accomplished by referring to the following goals achieved in this study:

- The optimisation of the isolation and purification procedure for ovine liver microsomal cytochrome b₅ in order to obtain sufficient material for aggregation and immunological studies.
- The removal of the membrane binding domain of cytochrome b₅ by means of tryptic digestion to establish the role of the carboxyl terminal in ovine cytochrome b₅ aggregation.
- The raising of antibodies against both the trypsin truncated and intact forms of cytochrome b₅ to study the aggregation of the protein.
- The investigation into the influence of purified cytochrome b₅ on steroidogenesis in ovine adrenal microsomes.

OPSOMMING

Die isolering en karakterisering van skaaplewersitochroom b_5 , soos beskryf in hierdie proefskrif, is uitgevoer deur die volgende doelwitte suksesvol af te handel:

- Die optimalisering van die prosedure vir die suksesvolle isolering en suiwering van skaaplewersitochroom b_5 ten einde genoegsame hoeveelhede van die suiwer proteïen te hê vir die bestudering van die aggregasie van die proteïen sowel as 'n immunologiese studie.
- Die verwydering van die membraanbindingsdomein van sitochroom b_5 om die invloed van die karboksielterminaal op die aggregering van die proteïen te bestudeer.
- Die gebruik van sowel die tripties gesnyde as die intakte vorms van sitochroom b_5 om 'n immuunrespons in hase op te wek vir die verkryging van sitochroom b_5 spesifieke anti-ligggame.
- Die gebruik van die gesuiwerde proteïene om die invloed van sitochroom b_5 op adrenale steroïdogenese te bestudeer.

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ABBREVIATIONS

3 β HSD	3 β -hydroxy-steroid dehydrogenase isomerase
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
BAEE	α -Benzoyl-L-arginine ethyl ester
BCA	Bicinchoninic acid
cDNA	Complementary deoxyribonucleic acid
CMP	Cytidine monophosphate
CRFH	Corticotropin releasing hormone
CYP17	Cytochrome P450c17
DEAE	Diethyl amino ethyl
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid
DOC	Deoxycorticosterone
DOCL	Deoxycortisol
EDTA	Ethylenediaminetetraacetic acid
ESMS	Electrospray mass spectrometry
FAD	Flavin adenine dinucleotide (oxidised)
FMN	Flavin mononucleotide
FRET	Fluorescence resonance energy transfer
HDL	High density lipoprotein
HMGCoA	3-Hydroxy-3-methylglutaryl coenzyme A
HPGPC	High performance gelpermeation chromatography
HPLC	High performance liquid chromatography

LDL	Low density lipoprotein
LSIMS	Liquid secondary ion mass spectrometry
mRNA	Messenger ribonucleic acid
MS/MS	Tandem mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenosine dinucleotide phosphate
NMR	Nuclear magnetic resonance spectrometry
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Q-ToF MS	Quadropole-time of flight mass spectrometry
SCP-2	Sterol carrier protein 2
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
StAR	Steroidogenic acute regulatory protein
TPCK	L-(1-Tosylamido-2-phenyl)ethylchloromethyl ketone
Tris	Tris(hydroxymethyl)-aminomethane

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CHAPTER 1

INTRODUCTION

Cytochrome b_5 was first observed in 1940 in liver homogenates as a cytochrome with a broad α -peak in the region of 560nm (1). This new cytochrome was initially designated cytochrome b_1 , but was later renamed by Strittmatter and Ball (2) to cytochrome m (microsomes) as the pigment was detected in relative high concentrations in liver microsomes (2). In 1950, a unique cytochrome was also detected in cecropia silkworm larvae (3). This pigment was later designated cytochrome b_5 by Pappenheimer and Williams (4), who concluded from the position of the absorption bands that this cytochrome was the same as the cytochrome observed by Keilin *et al.* in liver homogenates. Several years later, in 1956, the soluble form of cytochrome b_5 was isolated and partially characterised by Velick and Strittmatter (5).

Cytochrome b_5 , an electron carrier for several oxygenases, is a small heme containing protein that is found in both a soluble form, necessary for its function in the erythrocytes, and a membrane bound form found in the endoplasmic reticulum and mitochondria (6). Endoplasmic reticula of all cells in the body contain cytochrome b_5 , but the highest levels are found in liver cells where cytochrome b_5 participates in various biochemical reactions, such as lipid biosynthesis, hepatic cholesterologenesis and N-glycolylneuraminic acid biosynthesis (6). The hemoprotein is reduced by cytochrome b_5 reductase with electrons derived from NADH or from NADPH via cytochrome P450 reductase (7). It has been shown that the presence of cytochrome b_5 in the testis and adrenal gland also plays an important role in steroidogenesis (8, 9). In the adrenal gland the protein can either inhibit or enhance certain reactions through direct electron transfer or through an allosteric interaction with cytochrome P450. Abnormalities in the levels of cytochrome b_5 in any of the above mentioned cells can lead to serious disorders, ranging from methemoglobinemia to Cushing's syndrome (10, 11).

Recombinant technology offers an attractive option to produce of cytochrome b_5 for the investigation of the physiological influences of the protein on cellular processes. Doeblner *et al.* (12) investigated the self association of cytochrome b_5 in phosphatidylcholine vesicles. Purified recombinant mutant cytochrome b_5 was used in this study and he showed that self association of cytochrome b_5 in a phospholipid double membrane does not occur. During his studies into the influence of cytochrome b_5 on the lyase activity of cytochrome P450c17, Miller *et al.* (13, 14) also used a recombinant protein. Initial studies of sheep liver cytochrome b_5 in our laboratory contradicted the work published by Doeblner *et al.* (12). This

observation of self-assembly prompted us to isolate and characterise native cytochrome b_5 from sheep liver to further elucidate the self association of cytochrome b_5 and to determine the physiological significance, if any, of this association. The influence of purified full length and trypsin truncated cytochrome b_5 on ovine adrenal steroidogenesis was also investigated.

Sequences for cytochrome b_5 have been determined for a number of species that have been studied, as shown in Chapter 2. These studies highlighted the wide range of activities that this ubiquitous hemoprotein has exhibited in different mammalian species. In our laboratory, ovine cytochrome b_5 , and its influence on ovine steroidogenesis, has been under investigation for a number of years. Initial investigation of the structural properties of ovine liver cytochrome b_5 in our laboratory indicated that protein occurs mainly as an aggregate with a relative molecular mass of $\sim 60,000$ Da. In an effort to dissociate the aggregate, various methods were utilised of which a lowering of the pH from 8.1 to 5.2 was the most effective. Immunoblots, using an antibody prepared against a monomeric isolate of cytochrome b_5 , showed that the antibody recognised only the aggregated form of the hemoprotein. The same antiserum, when applied in immunohistochemical studies, showed that cytochrome b_5 was concentrated in the cytoplasm of the cells of the *zona fasciculata* of the sheep adrenal cortex (15).

Further experiments focused on the solubilization of the aggregated cytochrome b_5 to obtain the monomeric form of microsomal cytochrome b_5 . The membrane anchoring domain was subsequently removed by means of controlled tryptic digestion. A product was obtained that was smaller in size, but still retained its spectrophotometric properties. From this study evidence was obtained that the hydrophobic membrane anchoring domain was involved in the observed aggregation (16). This study utilised methods of Strittmatter *et al.* (17) and Omura and Takesue (18) for the preparation of cytochrome b_5 and the tryptic truncated form of the protein from ovine liver. The yields from these methods were low and the results were not reproducible. It was therefore not possible to make any definitive deduction about the aggregation state of ovine microsomal cytochrome b_5 . A number of questions therefore remain unanswered, of which the most intriguing was the aggregation state of ovine cytochrome b_5 *in vivo* and the influence of this aggregation on the physiological role of cytochrome b_5 in adrenal steroidogenesis.

The current study was initiated with the following aims in mind:

1. to gain a thorough knowledge of the structure and functions of cytochrome b_5 from available literature;
2. to optimise the isolation and purification procedure for ovine liver microsomal cytochrome b_5 in order to obtain sufficient material for aggregation and immunological studies;
3. to remove the membrane binding domain of cytochrome b_5 by means of tryptic digestion to establish the role of the carboxy terminal in ovine cytochrome b_5 aggregation;
4. to raise antibodies against both the trypsin truncated and intact forms of cytochrome b_5 to study the aggregation of the protein; and
5. to investigate the influence of purified cytochrome b_5 on steroidogenesis in ovine adrenal microsomes.

In Chapter 2 a review of the current knowledge of the structure, function and properties of cytochrome b_5 is given as background for the study.

An alternative method for the isolation of ovine liver cytochrome b_5 and the preparation of trypsin truncated cytochrome b_5 was developed in this study. This method used to isolate the catalytic domain of cytochrome b_5 , produced a significant higher yield of active truncated protein than isolation procedures used previously. These methods and the results obtained, are discussed in Chapter 3.

An investigation into the aggregation states of ovine cytochrome b_5 is described in Chapter 4. The molecular mass of the purified ovine liver cytochrome b_5 was 15,260 Da as determined by electrospray mass spectrometry (ESMS). No other proteins with a higher or a lower molecular mass were observed indicating a pure preparation. SDS-Polyacrylamide gel electrophoresis (SDS PAGE), however, indicated multimeric forms of the protein, even after stringent detergent and mercaptoethanol pre-treatment. Trypsin treatment of cytochrome b_5 resulted in a truncated enzyme with a molecular mass of $\pm 10,000$ Da. The aggregation of cytochrome b_5 was abolished by the tryptic removal of the membrane binding region.

High performance gel permeation chromatography (HPGPC) of the purified full length cytochrome b_5 showed two distinct molecular species, a monomer and a tetramer. The

equilibrium between the monomeric and tetrameric form of the enzyme could be manipulated by changes in the ionic strength of the elution buffer. In Western blot analyses antibodies against the truncated protein recognised only the low molecular mass truncated form of the protein and not the full length cytochrome b_5 , or any of the higher molecular mass complexes. This result showed the involvement of the membrane binding domain of the protein, not only in aggregation, but also in the quaternary structure which determined epitope presentation for antibody production. Immunoblot analyses and immunocytochemistry of adrenal tissue, using antibodies against the full length cytochrome b_5 , indicated that the tetrameric form of the protein was in all probability the dominant specie *in vivo*.

Serum containing antibodies raised against both the truncated and intact forms of ovine cytochrome b_5 was used in the above mentioned investigations. The antibody serum against the intact form of the protein recognised the aggregated as well as the monomeric form of the protein. A method was developed to separate antibodies recognising the tetrameric form of the protein from antibodies recognising the monomeric form. Western blot analyses of purified trypsin truncated and native cytochrome b_5 as well as ovine adrenal, liver and testicular microsomes showed that the tetrameric form of the protein prevailed *in vivo*. The investigation into the aggregation of cytochrome b_5 is presented in Chapter 5.

Cytochrome b_5 has been shown to influence the 17,20-lyase activity of cytochrome P450c17 *in vitro*, apparently not through normal electron transfer. An allosteric mechanism for this action of cytochrome b_5 has been proposed by Auchus *et al.* (13). Chapter 6 deals with the results obtained from our study into the effect of purified cytochrome b_5 on ovine adrenal steroidogenesis thus addressing our final aim. The results indicate that cytochrome b_5 plays a role in the regulation of the 17,20-lyase activity of cytochrome P450c17 in ovine adrenal steroidogenesis.

In the final chapter conclusions are drawn from the results obtained in this study, and the relevance and implications of these results are discussed.

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CHAPTER 2

CYTOCHROME b₅: STRUCTURE, PROPERTIES AND FUNCTIONS

1.1 INTRODUCTION

Cytochrome b₅ is a small electron transfer hemoprotein and, in conjunction with cytochrome b₅ reductase, is capable of transferring or accepting a single electron originating from NADH (1). It exists as both a soluble cytosolic protein and an amphipathic membrane-bound protein. The main function of the soluble form is to maintain the oxygen carrying capacity of hemoglobin *in vivo* by keeping it in a reduced state (2). A second form of cytochrome b₅ was isolated from the mitochondrial outer membrane by proteolytic digestion (3), but the amphipathic properties of cytochrome b₅ was only recognised when it was later purified in an intact form in the presence of detergents (4, 5).

Although the membrane-bound form exists at low levels in the endoplasmic reticulum of all cells, the highest concentration is observed in the liver, where it provides reducing equivalents in various biochemical reactions (6). Cytochrome b₅ provides electrons for the desaturases responsible for lipid biosynthesis. Cytochrome b₅, together with cytochrome b₅ reductase, was shown to be essential for the elongation reaction of N-glycolyleuraminic acid by CMP-N-glycolyleuraminic acid hydroxylase (7). Reducing equivalents for the oxidation of lanosterol, a cholesterol precursor, are provided by cytochrome b₅ (8).

Due to its pivotal role in physiological and biochemical processes, cytochrome b₅ was the subject of numerous studies from as early as the 1940's. These investigations were hampered by the amphipathic character of the protein as well as the low solubility of the full length membrane bound form. Investigations have been facilitated by the availability of the recombinant protein. The bovine form was the first cytochrome b₅ gene to be isolated and characterised. Crystallisation of cytochrome b₅, together with amino acid residue alignments, permitted the identification and characterisation of the heme- and membrane-binding domains of the full length protein. The unique spectral properties as well as the stability of cytochrome b₅ makes it an ideal model for membrane bound protein.

1.2 THE STRUCTURE OF CYTOCHROME b_5

2.2.1 *Genes encoding cytochrome b_5*

The soluble erythrocyte cytochrome b_5 is very similar in amino acid sequence to the hydrophilic part of the liver cytochrome b_5 . It has thus been suggested that the erythrocyte form is deduced from the liver form by proteolytic action during erythroid maturation. This hypothesis was experimentally supported by treating liver cytochrome b_5 with lysosomal proteases to yield two hydrophilic segments, one of which was indistinguishable from the erythrocyte form (9).

It was found that the sequences of the erythrocyte form were identical to the liver form, except for the C-terminal residue when the amino acid sequences of the erythrocyte cytochrome b_5 of human, rabbit and porcine origin were compared to the liver cytochrome b_5 of the same species. The residue at the end of the C-terminal of the erythrocyte form was found to be proline in human and rabbit cytochrome b_5 while for porcine it was a serine residue. A threonine residue was found at the C-terminal end in the three species' corresponding liver forms. This led to the suggestion that the two molecules are encoded by different mRNA species (10). Giordano *et al.* (11) suggested that the two forms of cytochrome b_5 are derived from two almost identical genes or from a common precursor mRNA through alternative splicing mechanisms.

It was found that the chicken DNA sequence was the same as that from liver cells when the cDNA clone encoding for erythrocyte cytochrome b_5 was isolated. The erythrocyte clone also encoded a protein that was exactly the same as the liver form, complete with a hydrophobic tail-region. These results led to the deduction that both forms are transcribed from a single gene, with the erythrocyte form resulting from proteolytic processing. The difference in the C-terminal residue between the two forms suggest the possible existence of a post-translational modification (12).

mRNA encoding for soluble rabbit cytochrome b_5 was demonstrated to be homologous to the mRNA of the liver form, but due to the inclusion of an internal exon, was 24 bp longer. Although the mRNA for the soluble form is longer, it encodes a smaller protein. This is due to the presence of a termination codon in the additional exon. It was therefore suggested that the mRNAs for the soluble and membrane-bound forms are derived from a common precursor mRNA through alternative splicing mechanisms (11).

The bovine form was the first cytochrome b_5 gene to be isolated and characterised (figure 2.1). It showed that the gene is divided into six exons, including the non-functional exon 4, and span about 28 kb. Consistent with other housekeeping genes, a TATA box is absent in the 5' flanking region while it contains two CAAT boxes and several G:C-rich SpI motifs. The general size and organisation of the gene is comparable with that of the human and rabbit genes. No sequence coding for soluble cytochrome b_5 could be found in reticulocyte mRNA but evidence for a transcribed pseudogene exists. It was thus concluded for bovine cytochrome b_5 that the soluble form is derived from the membrane-bound form by a post-translational modification mechanism (13).

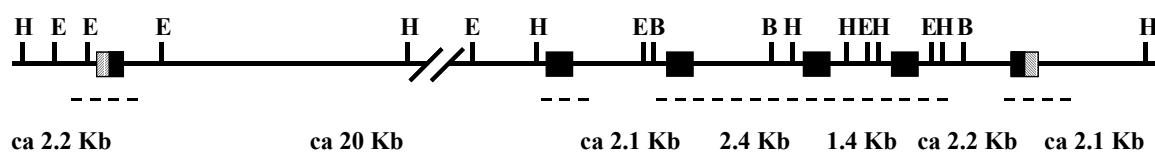


Figure 2.1 Restriction map of the bovine cytochrome b_5 gene. The hatched regions represent 5' and 3' nontranslated sequences while the broken line represents the region of the gene that has been sequenced. E: EcoRI H: HindIII B: BglII (11).

The human gene for cytochrome b_5 was mapped to chromosome 18. One of its pseudogenes was mapped to the X-chromosome. This was done by means of restriction enzyme digestion of PCR-amplified DNA (14).

2.2.2 Primary structure

In most mammals, liver cytochrome b_5 is membrane bound comprising of 134 amino acids, whereas soluble erythrocyte cytochrome b_5 comprises of 98 amino acids (15). The two forms are however indistinguishable in catalytic and spectral properties (16).

Initial isolations of cytochrome b_5 from rabbit liver microsomes were achieved by pancreatic lipase solubilization of the microsomes (17). In an adapted method, in the presence of trypsin inhibitor, what was believed to be two different forms of cytochrome b_5 was isolated from calf liver. These two forms differed in that one contained a carboxyl-terminal glutamylserine peptide sequence which was absent in the other. Tryptic digestion of either one of the two proteins yielded the same core heme peptide by cleaving two peptides off each protein from both the carboxyl-terminal and the amino-terminal ends (18).

A cytochrome b_5 that was larger than the trypsin- or lipase-extracted enzyme was obtained from rabbit liver microsomes when detergents instead of lipase were used to solubilise

proteins. The detergent solubilized cytochrome b_5 contained an additional 40 amino acids, which formed an extremely hydrophobic appendage. The presence of this hydrophobic appendage caused cytochrome b_5 to aggregate when placed in an aqueous environment in the absence of detergent (5).

Cytochrome b_5 isolated from human and porcine erythrocytes consist of 97 amino acid residues. The tryptic digested form, without its hydrophobic membrane binding domain, contain about 90 residues, while intact porcine cytochrome b_5 contain 133 residues (16). A comparison between rabbit and calf liver microsomal cytochrome b_5 showed that the rabbit cytochrome was 4 residues longer at the NH_2 terminal and differed with respect to 10 other residues (19).

It was noted by Ozols (20) that the sequence of the cytosolic heme-binding domain, residues 1-96, was highly conserved. The different forms of cytochrome b_5 all show a high degree of sequence identity. In a review by Schenkman *et al.* (21), 15 different forms of cytochrome b_5 from different phyla were compared. For all 15 forms, 12 of the ~134 amino acid residues showed complete identity in alignment (19 if the bacterial alignment is not included). The different mammalian species showed over 80% identity. The carboxy-terminal domains on the other hand showed a somewhat lower identity (figure 2.2).

2.2.3 Secondary and tertiary structure

Cytochrome b_5 is a small cylindrical acidic membrane protein consisting of 6 helices and 5 β -strands. The protein is folded into two domains, the larger of which is the cytosolic heme-containing, amino-terminal, hydrophilic region. The smaller region is the hydrophobic, membrane-binding, carboxyl portion of 14-18 residues. This domain is connected to the globular domain by a proline-containing hinge region of ~7 amino acid residues and followed by 7 polar amino acid residues (21, 22). Proteases can clip the protein before or after the hinge region.

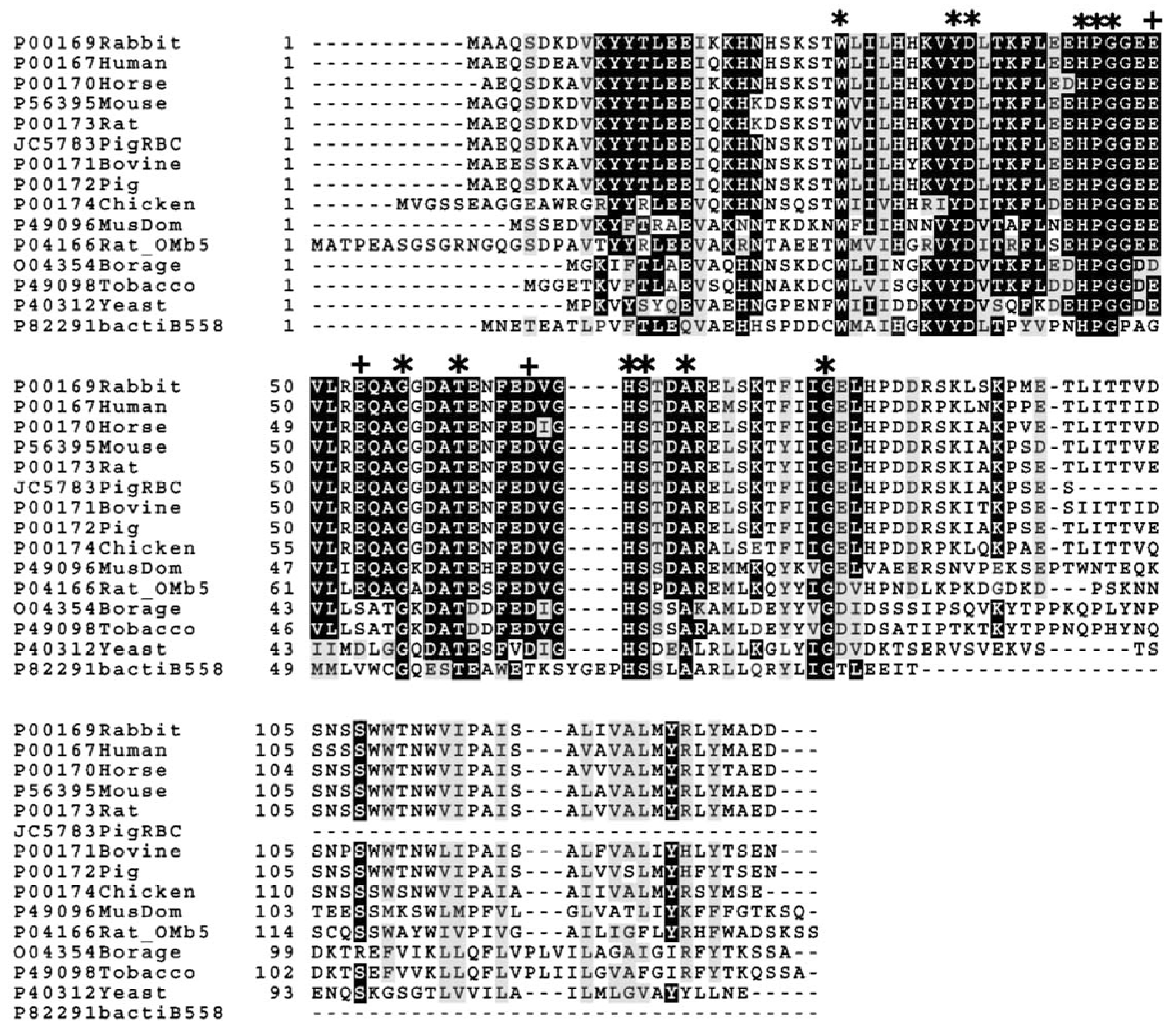


Figure 2.2 Alignment of cytochrome b₅ from different phyla. Accession numbers are provided for the various forms of cytochrome b₅. Forms include bacteria (bacti), tobacco plant, housefly (MusDom), rat mitochondria outer membrane (Rat_OMb5) and erythrocyte soluble protein (RBCsolpi). Asterisks above alignments indicate fully conserved residues; plus signs above alignment indicate acidic residues implicated in charge-pairing interactions with redox partners. Black indicates identical residues for a position, gray indicates a conservative substitution (such as an aspartate for glutamate or lysine for arginine) (19).

2.2.3.1 Heme binding domain. The hydrophilic domain of cytochrome b₅ contains a non-covalently bound heme group. The heme is located in a hydrophobic pocket. Two highly conserved histidine sidechains, His44 and His68 (figure 2.2), co-ordinate the heme-iron. The heme is inserted in a cleft, between 4 α -helices, on the catalytic region with a heme edge exposed to the environment (figure 2.3) (21, 23).

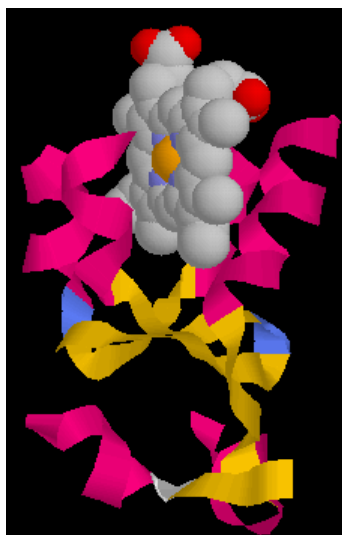


Figure 2.3 Computer model (RasMol) of the structure of cytochrome b₅ showing the 4 α -helices (pink) surrounding the heme.

A hydrophobic patch of 350 Å² is found on the surface of cytochrome b₅. This is an exception to the rule that hydrophobic residues are primarily grouped in the interior of a protein molecule (24). The hydrophobic patch, surrounded by negatively charged residues, consists of amino acid residues, Phe40, Pro45, Leu75 and Phe79 as depicted in figure 2.2. Except for residue Phe79 which is conservatively substituted for tyrosine, this patch is totally conserved among different species.

Phe/Tyr79 is partially exposed to the surface of cytochrome b₅ and its buried surface contacts the heme crevice (23). It has been claimed that this hydrophobic patch could be the second electron transfer site of cytochrome b₅ with its amphipathic redox partners although it has been proven that Tyr79 is not necessary for the binding and electron transfer function. Nor is it a determinant of the reduction potential or spectroscopic properties of cytochrome b₅. Rather, it seems to shield the heme from solvent and thus enhances the association of the heme with the polypeptide chain of cytochrome b₅ (25).

The Phe40 residue, which is closer to the heme than the Phe/Tyr79, plays an important role in the stability of the protein. If the hydrophobic phenylalanine residue is replaced with a slightly more hydrophilic hydroxyphenyl or imidazole, it disturbs the hydrophobicity of the heme pocket, having a marked effect on the redox potential (26).

Several acidic residues around the exposed heme edge as well as one of the heme propionate residues, protrude into a common plane in the solvent. These anionic residues are Glu49, Glu53 and Asp65 (figure 2.2), of which residues Glu49 and Asp65 are highly conserved in

all species examined, except in bacteria. These residues have been implicated in charge-pairing interactions between cytochrome b_5 and other electron transfer proteins, indicating electrostatic forces stabilising the formation of redox complexes with cytochrome b_5 (21).

2.2.3.2 Membrane-binding domain. Although not as highly conserved as the heme-binding domain, considerable identity exists in the tail region, especially in the mammalian proteins (27). The carboxyl termini of all cytochrome b_5 proteins investigated so far exhibit a hydrophobic stretch of ~23 residues with 10 residues at the carboxy terminal which are thought to play an important role in membrane binding. It was in fact shown that these 10 residues are necessary for the targeting of cytochrome b_5 to the endoplasmic reticulum (28).

High levels of free energy are required to remove charged and polar amino acid residues from an aqueous environment and to bury the residues in a hydrophobic lipid bilayer. Charged and polar residues will bind to the charged head groups of phospholipids in the endoplasmic reticulum. In order to remove a polypeptide from an aqueous environment and insert it into a hydrophobic lipid bilayer, the amino and carbonyl groups of the protein must be maximally hydrogen bonded to one another to favour the negative change in free energy required for insertion. A protein in the hydrophobic core of a membrane would be stable as either an α -helix or as a β -barrel, as these are the simplest and energetically the most stable structures, with all backbone amino and carbonyl groups hydrogen bonded to one another. In contrast, in a two-stranded anti-parallel β structure, only one-half of the carbonyl and amino groups are hydrogen bonded to one another. The structure with the highest number of hydrogen bonds and highest thermodynamic stability, is the α -helix. This led Singer (29) to conclude that the membrane-binding regions of proteins may largely be in an α -helical conformation.

Two different conformations for the membrane-binding domain of cytochrome b_5 have been proposed. One, a conventional transmembrane α -helix (30, 31) and the other a model where both the amino and carboxyl termini are on the cytosolic side of the membrane due to a hairpin loop in the α -helical hydrophobic segment (figure 2.4) (32, 33). This loop could occur at Pro116 (figure 2.2) of the hydrophobic segment. More recent work, however, tends to support the transmembrane model (34).

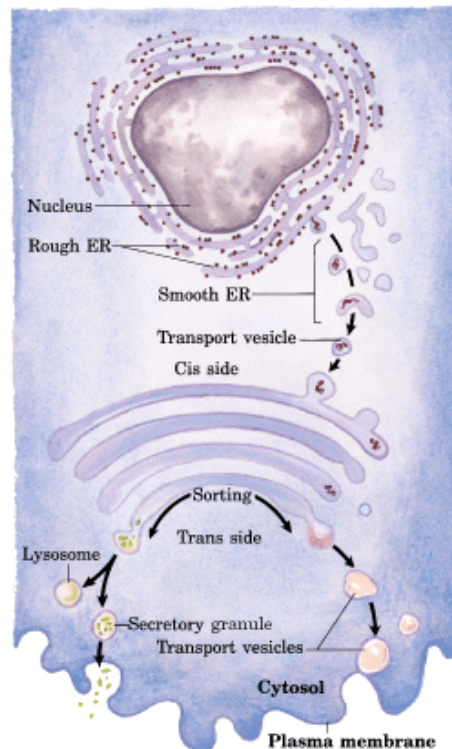


Figure 2.5 Endoplasmic reticulum and the Golgi complex. Proteins that enter the endoplasmic reticulum undergo the first steps of glycosylation at this point. These proteins are carried to the cis face of the Golgi complex by vesicles that bud off the endoplasmic reticulum. The vesicles that bud off the trans face of the Golgi complex form lysosomes, peroxisomes, glycosomes or migrate to the plasma membrane (reproduced from 42).

Three potential pathways for the targeting and incorporation of these proteins into the endoplasmic reticulum membrane were revealed by the analyses of *in vitro* systems:

- (i) the signal-recognition-particle/signal-recognition-particle receptor-dependent pathway (figure 2.6); (ii) the signal-recognition-particle/signal-recognition-particle receptor-independent but receptor-mediated pathway, and (iii) the receptor independent and hydrophobic insertion pathway (35, 41).

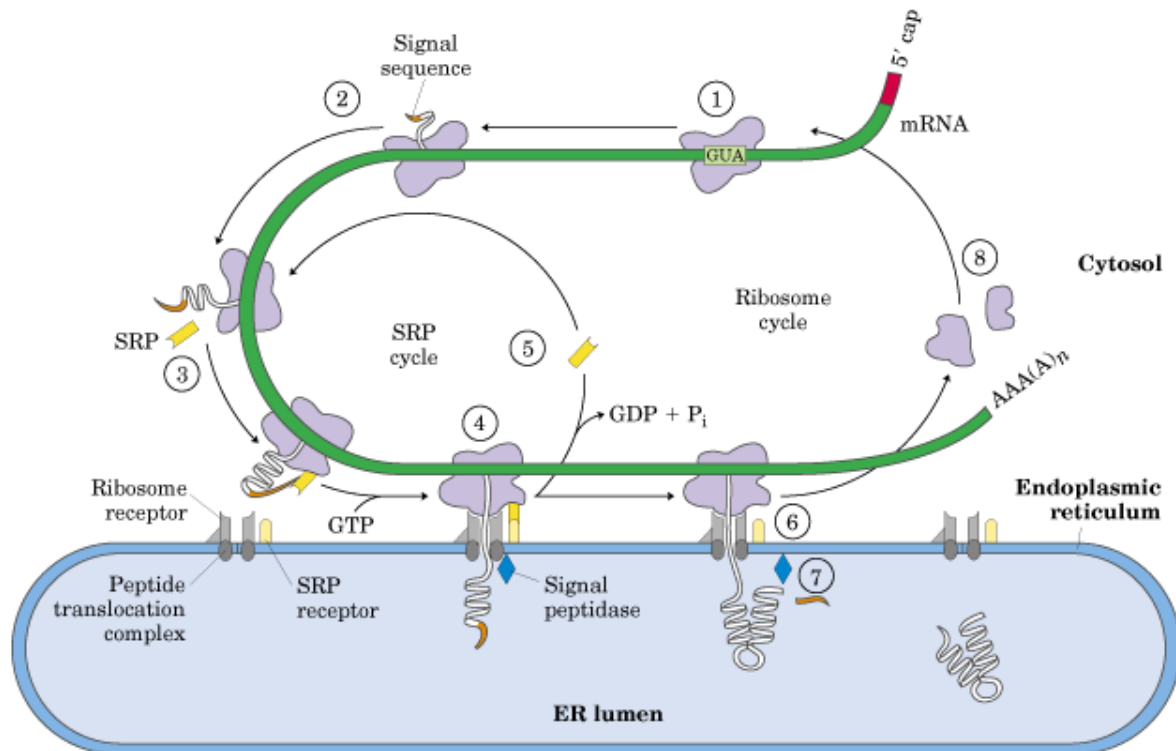


Figure 2.6 Sequence of events during the synthesis of proteins and their subsequent targeting to the endoplasmic reticulum membrane. 1: Ribosomal binding to the mRNA and initiation of translation; 2: The signal sequence is translated at the 5' end of the mRNA; 3: Recognition of signal sequence by signal recognition particle (SRP) and the temporarily halting of further translation; 4: The SRP recognizes and binds to the SRP receptor (docking protein). The signal sequence is inserted into the endoplasmic reticulum; 5: The SRP is released; 6: Protein synthesis is resumed and the polypeptide chain is pulled through the membrane; 7: Signal sequence is cleaved by signal peptidase. Proteins destined to remain in the endoplasmic reticulum have peptidase resistant signal sequences; 8: The protein enters the endoplasmic reticulum lumen and the ribosome is recycled (reproduced from 42).

Early studies into the incorporation of the membrane-bound form of cytochrome b_5 in the endoplasmic reticulum membrane concluded that the process is spontaneous and promiscuous, i.e. pathway (iii) (receptor independent and hydrophobic insertion pathway) (31, 33). This, according to more recent authors, will lead to intracellular chaos *in vivo*, as a specific membrane receptor is needed for *in vivo* binding of cytochrome b_5 to the endoplasmic reticulum membrane and the subsequent functioning with its membrane associated electron transfer partners (43).

Ten amino acid residues were identified on the C-terminal of cytochrome b_5 as necessary for targeting the endoplasmic reticulum (28). With these residues removed, targeting and binding of cytochrome b_5 to the endoplasmic reticulum membrane was abolished. When these ten

residues were coupled to foreign proteins, it was found that these proteins targeted the endoplasmic reticulum membrane (27). The proposed pathway for targeting and incorporation of cytochrome b_5 to the endoplasmic reticulum membrane is thus the 'signal-recognition-particle/signal-recognition-particle receptor-independent, but receptor-mediated' pathway.

A second type of membrane bound cytochrome b_5 exists. Mitochondrial cytochrome b_5 , which is analogous to the endoplasmic reticulum form, is associated with the mitochondrial outer membrane. Cytochrome b_5 possibly has another mechanism for targeting either the mitochondrial outer membrane or the endoplasmic reticulum membrane since one gene presumably encodes a membrane-bound form. Recent results obtained with mammalian cytochrome b_5 indicated that the net charge at the extreme of the C-terminal plays a role in targeting the protein to the membrane (44). The seven C-terminal residues of mammalian endoplasmic reticulum cytochrome b_5 were substituted with one or two arginine residues. This resulted in targeting of the mitochondrial outer membrane by the endoplasmic isoform. The insertion of a single threonine C-terminal residue also resulted in promiscuous localisation. A protein possessing a net positive charge will favour the mitochondrial outer membrane, while one with a net negative charge will favour the endoplasmic reticulum. Analyses of cytochrome b_5 sequences in the data bank revealed that only the mammalian endoplasmic reticulum isoform showed a characteristic negative charge in the extreme C-terminal portion (44). It is thus possible that in many organisms, cytochrome b_5 is a mitochondrial outer membrane protein, and not an endoplasmic reticulum protein. It was therefore concluded by Borgese *et al.* (44) that the endoplasmic reticulum and the mitochondrial outer membrane compete for the same polypeptide, while targeting occurs directly from the cytosol.

The question of the mechanism of residence was addressed by tagging two versions of cytochrome b_5 with potential N- and O-glycosylation (N-glyc and O-glyc) sites at the C-terminal extremity (45). The approach allowed discrimination between retention and retrieval. The assay, based on carbohydrate acquisition by O-glyc cytochrome b_5 , showed evidence that cytochrome b_5 gained access to enzymes which are localised in the Golgi complex and capable of catalysing the first steps of O-glycosylation. Although it is known that Golgi enzymes slowly recycle through the endoplasmic reticulum, those which are modified are not generally found in the endoplasmic reticulum. It is accepted that these

enzymes are inactive in the endoplasmic reticulum. Glycosylation could be inhibited by the addition of okadaic acid, a phosphatase inhibitor, which blocks transport out of the endoplasmic reticulum. If the Golgi enzymes were active in the endoplasmic reticulum, the addition of the inhibitor would not have been able to inhibit glycosylation due to the entrapment of the circulating enzymes. The N-linked oligosaccharide provided no evidence of retrieval from a downstream compartment. These results led to the suggestion that cytochrome b_5 recycles slowly between the endoplasmic reticulum and the *cis*-Golgi complex. Furthermore, it was suggested that the sorting of tail-anchored proteins involve both dynamic retrieval as well as retention (45).

1.3 PROPERTIES OF CYTOCHROME b_5

2.3.1 *Relative molecular mass*

Since the amino acid composition is highly conserved through all species, the relative molecular mass for cytochrome b_5 does not differ much among species. Initial studies suggested that the relative molecular mass of pig liver and calf liver cytochrome b_5 was 12,800 Da and 12,700 Da respectively (46, 47). These relative molecular masses were determined by means of ultracentrifugation. A relative molecular mass of 16,000 Da was obtained when cytochrome b_5 was isolated from calf livers using detergents at a low temperature to preserve the isolated proteins (48).

Reported relative molecular masses for different species were 16,900 Da for rabbit liver microsomal cytochrome b_5 , values between 15,000 Da and 17,000 Da for pig liver cytochrome b_5 and 14,400 Da to 16,000 Da for calf liver cytochrome b_5 (1, 48, 49). It was only when mass spectrometric analyses were employed that the relative molecular mass could be determined more accurately. The hydrophobic membrane binding domain of cytochrome b_5 was removed by means of tryptic digestion to enhance cytochrome b_5 solubility in an aqueous medium prior to analyses. Although the trypsin solubilized form of cytochrome b_5 was used, valuable information about the structure could be gathered due to the high accuracy of liquid secondary ion mass spectrometry (LSIMS) and tandem mass spectrometry (MS/MS) analyses (values determined within a 2 Da accuracy). Relative molecular masses for the solubilized forms of bovine and rabbit cytochrome b_5 were determined at $9,462.2 \pm 2$ Da and $9,502.3 \pm 2$ Da respectively. A second, larger form of $10,144.5 \pm 2$ Da was observed for the rabbit cytochrome b_5 which was attributed to a blocked amino-terminal residue (6).

2.3.2 *Stability of cytochrome b₅*

Cytochrome b₅ is stable at room temperature for days and indefinitely at -20°C (48). At a neutral pH, the heme dissociates partly from the protein at temperatures between 50-70°C. At temperatures higher than 70°C the heme dissociates completely. The dissociation reaction is reversible after a short heating period (50). Work done on intact bovine microsomal cytochrome b₅ showed that, as opposed to the single transition during thermal unfolding of the truncated hydrophilic protein, the intact protein exhibits two transitions. The midpoint temperature for the hydrophilic variant is around 73°C while the midpoint temperatures for the two transitions of the intact protein are around 43.4°C and 71°C (51). By the addition of a non-ionic detergent to intact cytochrome b₅ only one transition point was observed as opposed to the reported two.

At a neutral to slightly alkaline pH and ionic strength of 0.02 to 0.4, cytochrome b₅ forms aggregates. Ito and Sato (4) reported octameric aggregation complexes in aqueous solution which involve non-polar segments of the molecule. The heme peptide remains in the monomeric state under these conditions while the non-polar segment alone forms high molecular weight aggregates (48). At sufficiently high pH values, rearrangement of the heme ligands takes place which gives a product with increased high-spin characteristics which can be observed spectrophotometrically. This transition is reversible (46).

2.3.3 *Spectral properties*

There are three principal absorption bands in the absorption spectrum of many ferrohemoproteins, called the cytochrome absorption bands. The bands, the α -band, β -band and the γ -band (the Soret band), result from the absorption of radiation by the heme groups (52). Oxidised cytochrome b₅ exhibits an absorption maximum at 413nm in the Soret area. Reduced cytochrome b₅ shows absorption maxima at 556nm for the α -band, 526nm for the β -band and in the Soret region at 423nm for the γ -band (figure 2.7).

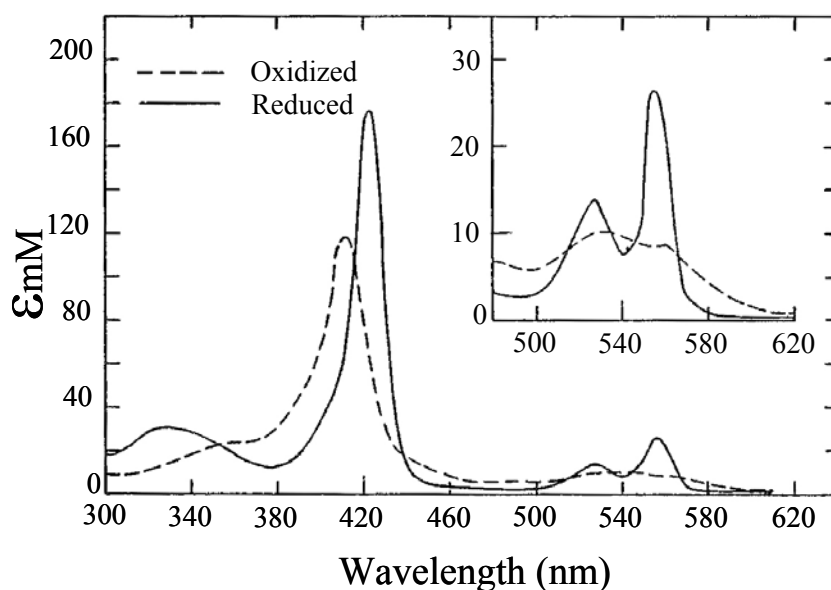


Figure 2.7 The absorption spectra of oxidised and reduced microsomal cytochrome b_5 (15).

A wide range of reducing agents can be used to obtain the same spectrum in the visible region. In a microsomal system, NADH is used as reducing agent in the presence of cytochrome b_5 reductase to distinguish cytochrome b_5 from the other cytochromes present in the solution (17, 49, 53). In a purified cytochrome b_5 sample, in the absence of reductase, it can be reduced with sodium dithionite. From the absorption values for the oxidised and reduced forms, the concentration of cytochrome b_5 in the solution can be determined by using the Beer-Lambert law. The Beer-Lambert states that the absorbance of a beam of collimated monochromatic radiation in a homogenous isotropic medium is proportional to the absorption path length and to the concentration of the absorbing species. The law may be expressed by the following relations:

$$\Delta A = \epsilon \ell c$$

Where ' ΔA ' represents the change in absorbance, ' ϵ ' the molar (decadic) absorption coefficient, ' ℓ ' the absorbance pathlength (in centimetre) and ' c ' the concentration of the substance (in molar) (52).

A microsomal suspension contains NADH cytochrome b_5 reductase and cytochromes other than just cytochrome b_5 , such as cytochrome P450. The suspension is therefore reduced with NADH as only cytochrome b_5 can utilise electrons originating from this electron donor. This results in a rapid reduction of cytochrome b_5 characterised by an increase in absorbance at 556nm and 426nm and a decrease of absorbance at 409nm. The cytochrome b_5 content of the

suspension may be calculated from difference in absorbance between 556nm and 575nm ($\Delta A_{556-575}$) and by applying the millimolar absorbance coefficient of 21. Alternatively for $\Delta A_{426-409}$ the millimolar absorbance coefficient of 185 can be used (53).

In solutions where the cytochrome b_5 is pure enough to record a spectrum of the oxidised form, the cytochrome b_5 concentration can be calculated by applying the molar absorbance coefficient of 117,000 to the absorbance at 423nm of the oxidised form. Another method would be to apply the molar absorbance coefficient of 100,000 to the difference in absorbance between the oxidised and reduced forms at 424nm (53).

The paramagnetic susceptibility at 20° shows that cytochrome b_5 has essentially low-spin characteristics in both the reduced and oxidised states. The electron spin resonance absorption of cytochrome b_5 in frozen aqueous solution, at a neutral pH, shows the characteristic features of an essentially low-spin compound with some high-spin contributions. An increase in the pH (≥ 11.3) disrupts the heme with a concomitant gradual change in the light absorption properties until a poor defined spectrum with a broad γ -band at 385nm and an increase in absorption at 610nm remains. As mentioned previously, this is due to the formation of a product with increased high-spin character (46).

2.3.4 Reduction potential of cytochrome b_5

The standard potential (E_o') for cytochrome b_5 has been determined at +0.02V at pH 7 from equilibrium experiments (54). According to biochemical convention, the more positive the value for E_o' , the more oxidising the compound. To put this value in perspective, it can be compared to the standard potential of $\text{NAD}^+/\text{NADH} + \text{H}^+$, which is -0.32V.

When the reduction potential of cytochrome b_5 was measured with potentiometric electrodes, lower values were obtained than the more positive values (40-100mV) measured with surface modified electrodes. Surface modified electrodes function on the basis of electrostatic interaction of the modified surface with the protein. Such electrodes were used by Rivera *et al.* (55) to determine the factors modulating the reduction potential of cytochrome b_5 . Observed positive shifts in the voltametric measurements were thought to originate from the complex formation between the modified surface of the electrode and cytochrome b_5 . This is due to the fact that such a complex will neutralise the charge on the heme propionate on the exposed heme edge and by excluding water from the complex interface, lower the dielectric constant of the exposed heme microenvironment, both factors which result in the

destabilisation of the positive charge on the ferric heme with respect to the neutral ferrous heme. These results indicate that similar shifts in reduction potential may occur when cytochrome b_5 form a complex with its redox partners, prior to electron transfer. They concluded that the reduction potential of cytochrome b_5 is modulated by the heme edge containing the exposed heme propionate, a heme methyl and a heme vinyl, all of which form part of the surface of cytochrome b_5 (55).

Interprotein complex formation immediately precedes electron transfer. The binding of cytochrome b_5 with its redox partners involves the cluster of negative charges around the heme on the surface of the heme-binding domain. An anionic ring is formed around the heme by carboxyl groups, including a heme propionate, which induce a dipole moment. This dipole moment orientates the cytochrome b_5 for effective interaction with the complementary, positively charged dipole of its redox partner. Three of the seven anionic residues on the cytochrome b_5 surface are used for binding to specific basic residues on the redox partners (56).

One of the first ^1H -NMR studies of cytochrome b_5 indicated that the solution structure of this hemoprotein differs from the crystal structure. The solution structure consists of a mixture of isomeric forms which differ from each other in the orientation of the heme-moieity by a 180° rotation about the axis through the α and γ meso-carbon atoms (57). The two equilibrium forms were designated A and B by Dangi *et al.* (58). They determined by NMR studies that the most significant differences between the two forms were the presence of a hydrogen bond between the 7-propionate and the Ser64 amide in the A form, but not in the B form as well as a displacement of the heme group by 0.9 \AA out of the binding pocket of the B form relative to the A form.

Factors which could contribute to the difference in reduction potentials in the bovine protein include the differences in orientation of the axial imidazoles and differences in hydrogen bond strength to the imidazoles (59). It would appear as though the major effector of the reduction potential is the absence of the hydrogen bond to the S64 amide in the B form. The absence of the hydrogen bond frees up the propionate to charge stabilise the iron in the oxidised state and thus lowering the reduction potential.

Although most of the work was done using trypsin-solubilised cytochrome b_5 , due to its thermal stability and high solubility, evidence exists for the independent folding of the hydrophilic and hydrophobic moieties in the intact molecule (60). It is thus unlikely that the hydrophobic tail-region will affect the heme exchange in the catalytic domain.

1.4 FUNCTIONS OF CYTOCHROME b_5

2.4.1. Lipid biosynthesis

2.4.1.1. Fatty acid desaturation. Cytochrome b_5 participates in the aerobic unsaturated fatty acid biosynthesis pathway in mammals. Electrons, which it received from NADH-cytochrome b_5 , are donated to desaturases, a microsomal, cyanide-sensitive, non-heme, iron-containing protein. These electrons are used to generate an electron-deficient activated oxygen species, which in turn removes electrons from the saturated hydrocarbon (figure 2.8).

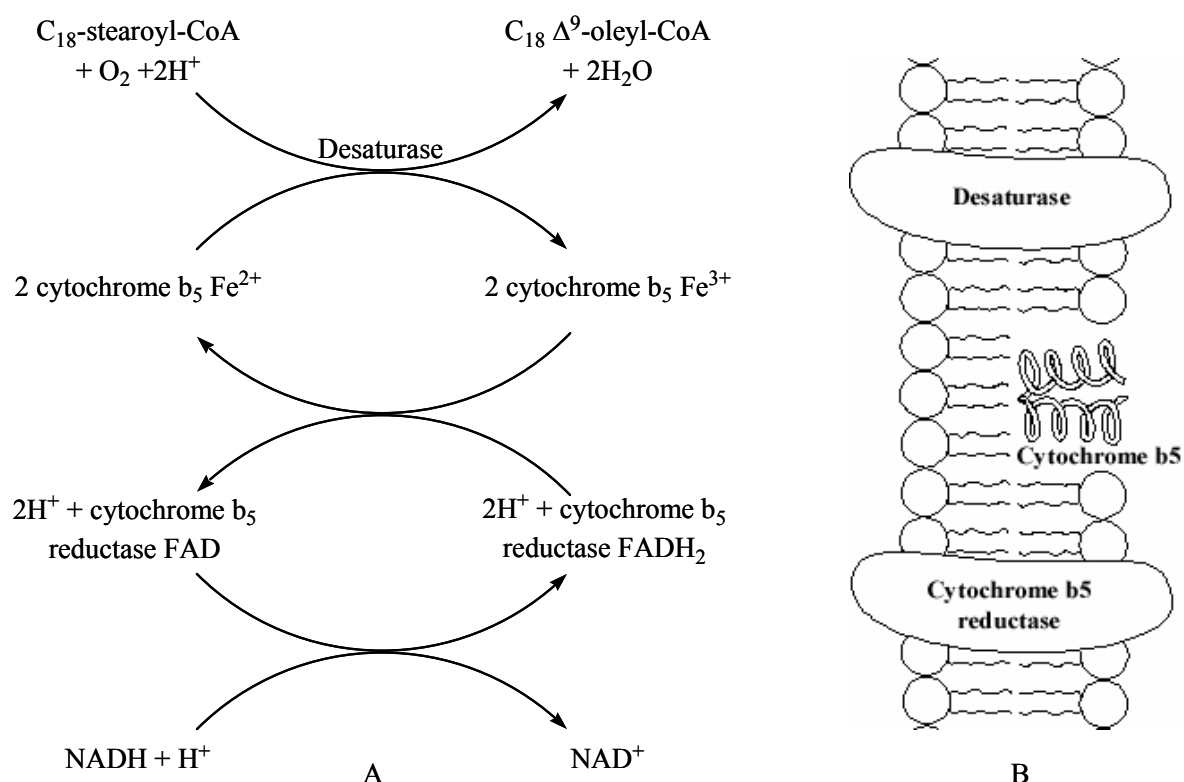


Figure 2.8 The fatty acid desaturase system. A: The flow of electrons from NADH to the desaturase enzyme. B: A model depicting the location of the proteins involved in the redox reactions in the membrane. (Redrawn 61)

As unsaturated fatty acids serve a structural role in cellular membrane fluidity, it is evident that enzymes that catalyse fatty acid desaturation play an important role in the control of membrane fluidity (62). Besides a structural role, unsaturated fatty acids are also the

precursors for polyunsaturated fatty acids, which in turn are the precursors for prostaglandins, thromboxanes and leukotrienes themselves.

Mammals possess at least three different microsomal fatty acid desaturases, namely Δ^5 , Δ^6 and Δ^9 desaturases. The Δ^5 desaturase is the least well characterised of the three. Cytochrome b_5 participation in this desaturase reaction can only be assumed due its requirement for NADH and oxygen. The Δ^5 desaturase introduces a double bond between C5 and C6 of C20 fatty acids, producing eicosatrienoic acid and arachidonic acid.

Okayasu *et al.* (63) confirmed the involvement of cytochrome b_5 in the Δ^6 desaturase reaction. The Δ^6 desaturase introduces a double bond between C6 and C7 of the C18 fatty acids, oleic acid, linoleic acid and linolenic acid. It has been suggested that Δ^5 and Δ^6 desaturase activities are present in the renal cortical microsomes, where they partially fulfil the kidney's need for arachidonic acid (64). During arachidonic acid synthesis in rat liver microsomes, the Δ^6 desaturation reaction is the rate-limiting step.

The Δ^{12} desaturation reaction catalyses for the formation of the essential fatty acids, linoleic and linolenic acid. In plants, where Δ^{12} desaturation takes place, cytochrome b_5 has been strongly implicated in the desaturation reaction. Antibodies to cytochrome b_5 were shown to inhibit the desaturation reaction by 93% in safflower microsomes (65).

2.4.1.2. Plasmalogen biosynthesis. Plasmalogens are a type of phospholipid in which the sn-1 position of the glycerol backbone bears a vinyl ether-linked fatty alcohol (an *O*-(1-alkenyl) group). Plasmalogens make up almost 20% of the phospholipid mass in humans and are found primarily within the ethanolamine phospholipid class. The final step in the biosynthesis of plasmalogen phospholipids, the plasmalogen form of ethanolamine phospholipids, is the insertion of the *cis*-double bond to form the vinyl ether functionality (figure 2.9).

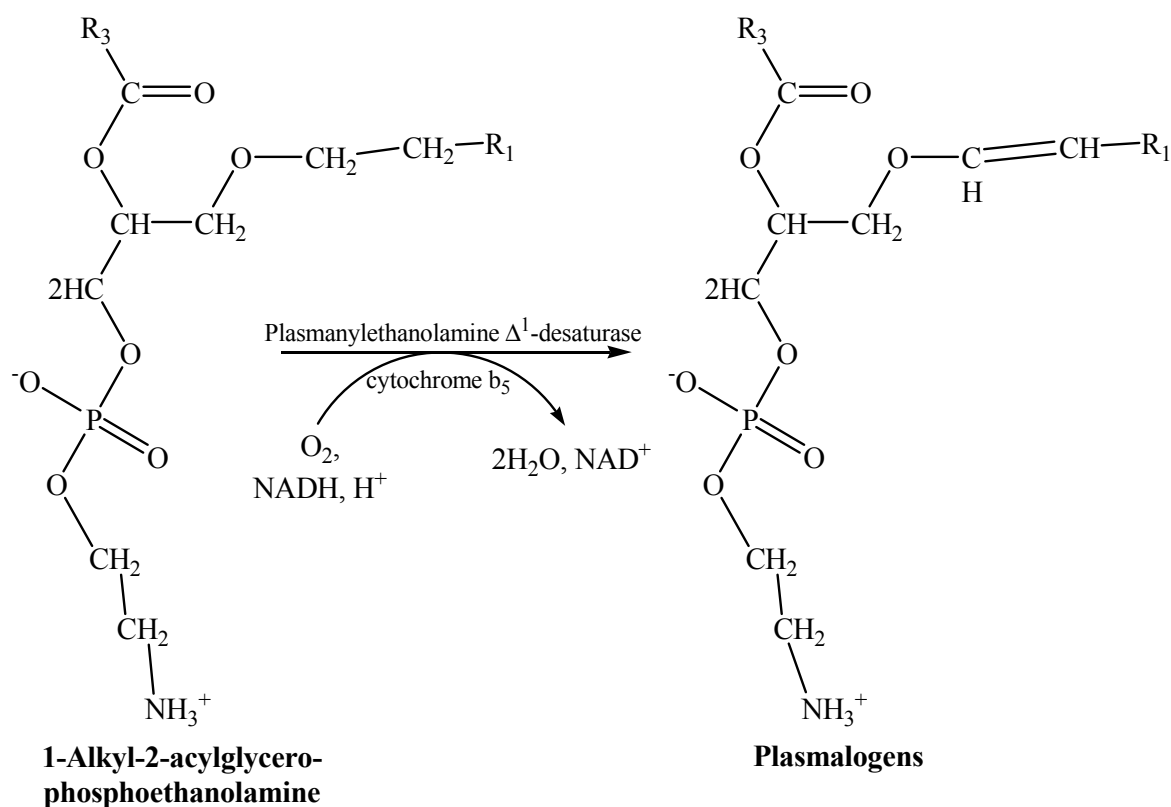


Figure 2.9 Synthesis of plasmalogens through the desaturation of 1-alkyl-2-acylglycerophosphoethanolamine to the vinyl ether. Electrons for the reaction are derived from NADH via cytochrome b_5 . (Redrawn 66)

Plasmalogen synthase, a cytochrome b_5 -dependent desaturase, is responsible for introducing the double bond α, β into the ether linkage (67). These phosphoglycerides are especially abundant in the membranes of muscle and nerve cells.

2.4.2. Hepatic cholesterologenesis

The desaturase that presumably oxidises the C-30 methyl group of lanosterol (a cholesterol precursor) to steroid-4 α -oic acid, is 4-methyl sterol oxidase. Treating liver microsomes with trypsin destroyed cytochrome b_5 with an observed loss in 4-methyl sterol oxidase activity. Adding purified cytochrome b_5 to these microsomes restored the 4-methyl sterol oxidase activity. These results indicate that reducing equivalents for this reaction are provided by cytochrome b_5 (8).

Another cytochrome b_5 dependent desaturase is lanosterol Δ^5 -desaturase. This enzyme introduces a double bond into lanosterol (Δ^7 -cholesterol, a cholesterol precursor) between C5 and C6 to produce 7-dehydrocholesterol (68).

2.4.3. Biosynthesis of N-glycolylneuraminic acid

N-glycolylneuraminic acid is the most common sialic acid in animals. It represents more than 95% of the total sialic acid in brain gangliosides. It was shown that, for the elongation reaction of N-glycolylneuraminic acid by CMP-N-glycolylneuraminic acid hydroxylase (figure 2.10), cytochrome b₅ and cytochrome b₅ reductase were essential (7).

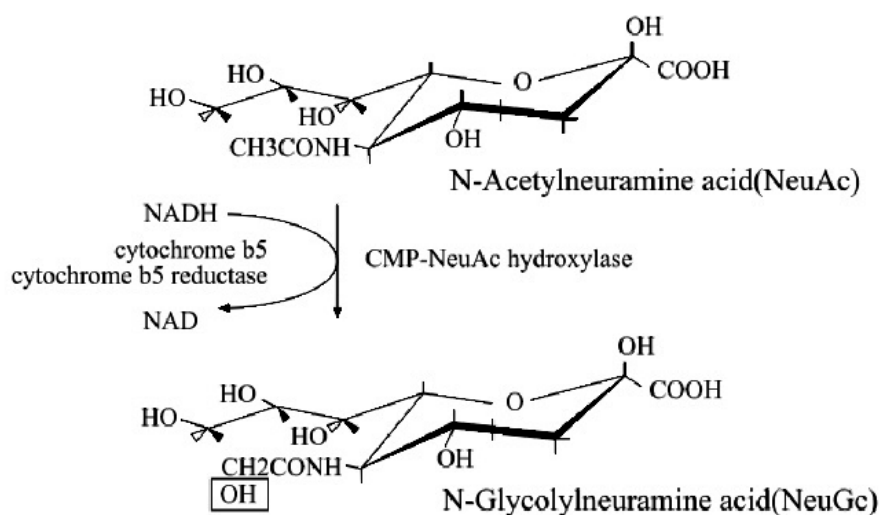


Figure 2.10 Involvement of cytochrome b₅ in the hydroxylation of N-acetylneuramine acid (NeuAc) by CMP-NeuAc hydroxylase to yield N-glycolylneuramine acid (NeuGc). (Redrawn 69)

2.4.4. Reduction of methemoglobin

In an adult human, about 3% of the circulating hemoglobin is oxidised to the non-functional ferric methemoglobin. It is generally accepted that red blood cells possess enzymatic reducing mechanisms to reduce the methemoglobin back to the ferrous state. Reducing equivalents are recruited from NADH via NADH-cytochrome b₅ reductase and cytochrome b₅ (70, 71).

NADH dehydrogenase, cytochrome b₅ reductase or methemoglobin reductase as it was also known, was originally shown to be a component of the system. A deficiency of this enzyme or structural abnormalities resulted in congenital methemoglobinemia. In cytochrome b₅ reductase reconstituted systems the methemoglobin reduction was slow in comparison with the rate of reduction in intact cells. The involvement of an additional component was therefore suggested. The reduction of methemoglobin by cytochrome b₅ reductase was stimulated markedly *in vitro* by cytochrome b₅ isolated from human erythrocyte cytosol. The

flow of electrons from NADH to methemoglobin can thus be described by the model depicted in figure 2.11.

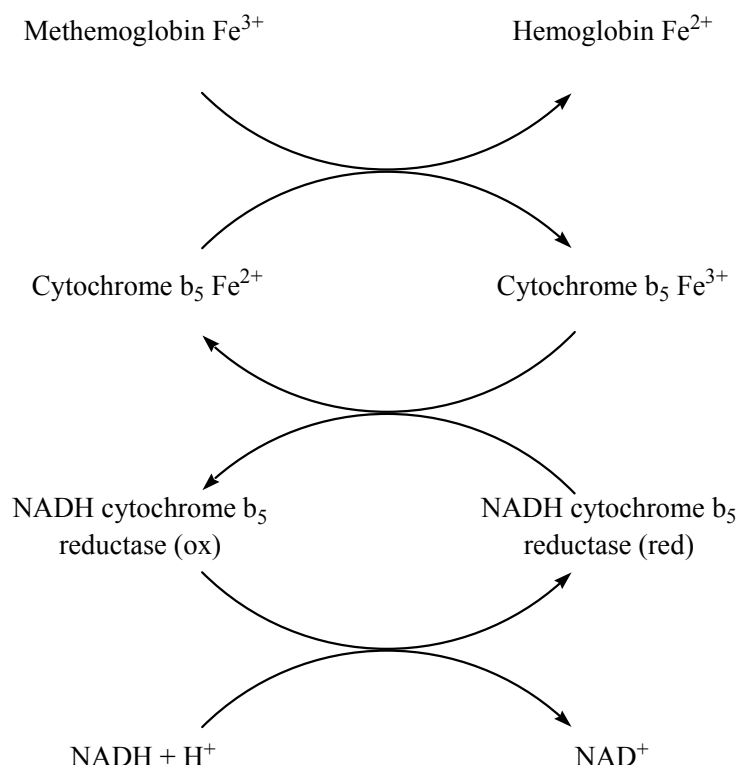


Figure 2.11 Redox reactions involved in the reduction of methemoglobin. NADH cytochrome b_5 reductase alone cannot support the reduction of methemoglobin cytochrome b_5 was shown to be an obligatory component in the effective reduction reaction. (Adapted from 71)

Qiao *et al.* (72) suggested that, contrary to the general paradigm, the acceptor and the donor protein do not have to bind in a specific complex before electron transfer can take place. The reaction between cytochrome b_5 and methemoglobin may simply involve bimolecular collision, as commonly found in small molecule electron transfer reactions. By aligning the dipoles prior to collision, electron transfer may be steered to take place effectively.

2.4.5. Reduction of cytochrome P450

The cytochromes P450 are mixed function oxidases which catalyse the oxidation of a wide range of compounds. Since the involvement of cytochrome b_5 in cytochrome P450-dependent reactions was suggested in 1971 (73), it has been a controversial issue. This stemmed from findings that certain cytochromes P450 were not stimulated by cytochrome b_5 while other researchers came to the conclusion that cytochrome b_5 was obligatory to a certain degree for the hydroxylation of certain substrates (21). As the cytochrome P450 monooxygenase reaction requires two electrons, it was suggested that while the first electron is provided by

cytochrome P450 reductase from NADPH, the second electron could be provided by cytochrome b_5 from NADH. To explain the mechanism of the cytochrome b_5 -imposed positive modifier action of the cytochrome P450 monooxygenase reaction, four suggestions have been made which will be discussed in more detail in Chapter 6 together with the influence of cytochrome b_5 on steroidogenesis (21, 74).

1.5 CONCLUSION

There are very few proteins that have such a profound effect on such a wide range of reactions in the living system as cytochrome b_5 . Although one soluble form and two membrane bound forms have been isolated and characterised to date, they all seem to originate from the same gene. The function of the soluble form to keep the hemoglobin in the reduced state is common through out all species. The hydrophilic region of the membrane bound forms show great homology with the soluble form of cytochrome b_5 as far as protein sequence is concerned. Functions of the mitochondrial outer membrane associated form also seem to be quite constant through all species. As far as the membrane-bound cytochrome b_5 form associated with the endoplasmic reticulum is concerned, many differences have been shown and reported. The most profound of these differences is found in its role in steroidogenesis. These differences, together with the fact that inconclusive results were obtained from isolated from tissue cytochrome b_5 , stimulated our interest to investigate these functions of cytochrome b_5 . With a specific interest in the role cytochrome b_5 plays in the adrenal steroidogenesis, it was necessary to first isolate and characterise cytochrome b_5 from an appropriate source. As the ovine liver form of this hemoprotein was previously investigated by one other group (75), sheep liver was chosen as a source of cytochrome b_5 for this study.

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CHAPTER 3

THE ISOLATION, PURIFICATION AND SOLUBILISATION OF CYTOCHROME b₅ FROM OVINE LIVER MICROSOMES

1.1 INTRODUCTION

As discussed in Chapter 2, cytochrome b₅ is an electron transfer heme protein that can accept and transfer single electrons in a number of biosynthetic pathways. It exists as a soluble cytosolic protein as well as an amphipathic membrane bound protein. In erythrocytes, the soluble form of cytochrome b₅ maintains methemoglobin in a reduced state while the membrane-bound form participates in various reactions that include lipid biosynthesis, cholesterol biosynthesis and steroidogenesis (3, 4, 5).

The availability of recombinant cytochrome b₅ has contributed greatly to the current knowledge of the protein. Although many studies into the physiological role and characterisation of cytochrome b₅ employed heterologous protein expression systems as a source of cytochrome b₅ (6, 7), the membrane binding domain of recombinant cytochrome b₅ is routinely modified to facilitate expression and subsequent purification. Yubisui *et al.* (7), for instance, could not express the membrane bound form of the protein, although the soluble form was successfully expressed and characterised. When Ito and Sato (8) initially isolated cytochrome b₅ from rabbit liver microsomes by means of detergent solubilization, they observed an aggregate which appeared to be an octamer. Western blot analyses, published by Lombard *et al.* (9), identified a high molecular mass protein when cytochrome b₅ was isolated from sheep liver. A relative molecular mass for ovine cytochrome b₅ of 16,200 ± 500 Da as determined by SDS PAGE was reported by Arinç *et al.* (1). During earlier studies in our laboratory, SDS PAGE analyses of a purified preparation showed an apparent aggregated form as well as a monomeric form of cytochrome b₅ with protein bands corresponding to molecular masses of 60,000 Da and 16,000 Da respectively. Electrospray mass spectrometry (ESMS) subsequently yielded an accurate molecular mass of 15,865 Da for ovine cytochrome b₅ (2). The molecular mass of 60,000 Da was approximately four times higher than the molecular mass of monomeric ovine cytochrome b₅. To date, no recombinant cytochrome b₅ has shown a tetrameric or octameric aggregate when analysed, though the native form of membrane bound cytochrome b₅ seems to exist as an aggregate. This raises the question

whether experimental data obtained with recombinant cytochrome b_5 would accurately reflect the physiological role of the protein *in vivo*.

An alternative method of isolation and purification was employed during our current study to determine whether the observed aggregation may result from the isolation procedure. The method was adapted from previously published methods and had a number of advantages when compared to established methods of cytochrome b_5 isolation. The hydrophobic membrane binding domain was removed by means of tryptic digestion under stringent conditions to elucidate the cause of cytochrome b_5 aggregation. The existing method for the removal of the hydrophobic domain of cytochrome b_5 has two inherent shortcomings – the yield of truncated cytochrome b_5 is low and the results are not reproducible when different types of trypsin are used.

Here we report an efficient, novel method for the successful isolation and purification of the intact form of cytochrome b_5 from sheep livers and the subsequent tryptic removal of the hydrophobic domain of the hemoprotein. The method consistently yielded high levels of truncated cytochrome b_5 .

1.2 MATERIALS AND METHODS

Materials

Unless otherwise specified, all chemicals used were of reagent grade and purchased from scientific supply houses. Pierce BCA kit was acquired from Pierce chemical company, Rockford Ill, USA. TPCK trypsin-sepharose, trypsin and sodium deoxycholate were acquired from Sigma, St Louis MO, USA. Whatman DE52 DEAE-cellulose resin and Triton x-100 were supplied by Merck, Darmstadt, Germany. Polyethylene glycol was purchased from Fluka, Sigma-Aldrich, St Louis MO, USA.

Preparation and detergent solubilisation of ovine adrenal microsomes.

Liver microsomes were prepared and fractionated using PEG as described by Yang and Cederbaum (10). Calcium chloride (CaCl_2) precipitated microsomes were prepared using a method adapted from Strittmatter *et al.* (11) The liver of a freshly slaughtered sheep was removed and kept on ice to minimise protein denaturing and protease activity. All subsequent steps were carried out at 4°C. After removal of connective tissue and fat, the liver was dissected into strips and homogenised in three parts 10 mM Tris-acetate buffer pH 8.1, containing 0.25 M sucrose and 0.1 mM EDTA, in a Waring blender at medium speed for 30

seconds. The homogenate was centrifuged at 13,000 g for 15 minutes after which the resulting supernatant was filtered through four layers of cheesecloth to remove excess solidified fat. The filtrate was diluted three times with cold distilled water, and 0.8 M CaCl_2 solution (1 ml/100 ml diluted filtrate) was added. After centrifugation of the suspension at 8,000 g for 5 minutes, the resultant microsomal pellet was resuspended in three parts 0.1 M Tris-acetate buffer, pH 8.1, containing 1 mM EDTA) in a Waring blender and dialysed overnight against 10 volumes 0.1 M Tris-acetate buffer, pH 8.1, containing 10 mM EDTA. Glycerol was added to the dialysate to a final concentration of 20% v/v and the resulting mixture was stirred for 1 hour. NADH (50 mg per litre) was added to the microsomal suspension before it was added to rapidly stirring acetone, at -7°C to -10°C . This mixture was stirred for 15 minutes and subsequently allowed to settle. The supernatant was siphoned off and the remaining acetone suspension centrifuged at 13,000 g for 10 minutes. The resulting pellet was resuspended in 0.1 M Tris-acetate buffer, pH 8.1, containing 1 mM EDTA, using a Waring blender. The centrifugation step was repeated and the washed pellet was resuspended in the same Tris buffer.

The microsomal preparations were solubilised as described by Strittmatter *et al.* (11). Triton X-100 was added to the microsomal suspension (2% final concentration) after which the suspension was stirred overnight and subsequently centrifuged at 15,000 g for 30 minutes. The supernatants were stored at -20°C until further use.

Purification of cytochrome b_5

The isolation and purification of ovine liver cytochrome b_5 was carried out as described by Swart *et al.* (12) using microsomes prepared by the CaCl_2 and PEG precipitation methods.

Removal of Triton X-100

The purified cytochrome b_5 obtained in the previous step was dialysed against ten volumes equilibration buffer (10mM Tris-acetate, pH 8.1, containing 0.1mM EDTA and 0.05% deoxycorticosterone) and applied to a DEAE-cellulose column (2 X 5cm). The column was extensively washed with equilibration buffer and the concentrated cytochrome b_5 subsequently eluted from the column with equilibration buffer containing 0.25M NaSCN. The purified cytochrome b_5 was dialysed against 10mM Tris-acetate buffer, pH 8.1, containing 10% glycerol and stored in aliquots at -80°C .

Tryptic digestion of intact purified cytochrome b₅

Purified cytochrome b₅, 0.5ml, was dialysed against ten volumes equilibration buffer and applied to a DEAE-cellulose column (1 X 5cm). The column was washed with 20ml equilibration buffer after which equilibration buffer containing 5000 BAEE (α -benzoyl-L-arginine ethyl ester) units of trypsin per millilitre buffer was circulated through the column at a flow rate of 27.5cm/hr for 3 hours. The procedure was repeated and the circulation time was extended to 24 hours. The column was washed with 50 ml equilibration buffer. The truncated cytochrome b₅ was eluted with equilibration buffer containing 0.25M NaSCN and subsequently dialysed against equilibration buffer.

High performance gel permeation chromatography (HPGPC)

HPGPC was performed on a Waters Hydrogel™ column connected to a Waters HPLC-system equipped with a Waters 991 photo diode array detector. A flow rate of 0.7ml/min was maintained for the mobile phase (10 mM Tris acetate pH 7.4) under conditions as described by Calabro *et al.* (13).

Electrospray Massspectrometry (ESMS) of purified cytochrome b₅

Cytochrome b₅ samples were analysed by ESMS under conditions as described by Swart *et al.* (12).

Quadropole-Time of Flight Mass Spectrometry (Q-ToF MS) of purified cytochrome b₅

Protein samples were prepared for analysis under denaturing and native conditions according to the following protocols. Stock protein solutions, 0.2 mg/ml, were prepared in acetonitrile/aqueous 1% formic acid (50/50) for denaturing conditions and in 25 mM ammonium bicarbonate for native conditions.

Positive ion electrospray MS data for the denatured protein samples were acquired using Q-ToF Micro and Q-ToF Ultima Global mass spectrometers fitted with nanoflow electrospray sources. For each analyte ~6 μ L of denatured protein solution was loaded into a borosilicate needle (Type F). The borosilicate needle was positioned in the nanoflow probe housing and the sample was infused into the mass spectrometer at a flow rate of ~10 nL min⁻¹. Positive ion electrospray MS data for the native protein samples were acquired using Q-ToF Micro and Q-ToF Ultima Global mass spectrometers fitted with standard electrospray sources. For each analyte, the protein was infused at 5 μ L min⁻¹ into the standard electrospray

source using the built-in syringe driver on the mass spectrometer. Both mass spectrometers were calibrated with a multipoint

Post acquisition processing using either the Max Ent or Transform software provided molecular mass information for the two samples. Max Ent 1 calculates the average mass of the species, while the Transform algorithm retains the isotopic distribution of the multiple charged ion species.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) analyses

SDS PAGE was carried out according to the method of Laemmli (14). Analyses of the protein samples, 5 µg, were done on a 15% resolving gel. Molecular weight markers (Rainbow[®] markers) were purchased from Pharmacia.

Non-denaturing polyacrylamide gel electrophoresis analyses

Non-denaturing polyacrylamide gel electrophoresis was carried out as described by Crambach *et al.* (15).

Cytochrome b₅ assay methods

The cytochrome b₅ content of microsomal preparations and purified fractions were determined spectrophotometrically as previously described (16) using a Beckman DU 650 spectrophotometer. A spectrum for oxidised cytochrome b₅ in 10 mM Tris-acetate buffer, pH 8.1 was recorded between 350 nm and 800 nm. The sample was reduced by the addition of NADH when reductase was present in the solution and with sodium dithionite in the absence of reductase and the reduced spectrum recorded as above. The content of cytochrome b₅ for the purified protein sample was calculated from the difference between the reduced and oxidised spectra at 424 nm (extinction coefficient, 100,000 M⁻¹cm⁻¹) (17). Microsomal cytochrome b₅ content was calculated from the reduced wavelength spectrum by applying a millimolar extinction coefficient of 185 to the absorbance change at 426 nm minus 409 nm ($\Delta A_{426-409}$).

Protein concentration assay

The protein concentrations of all samples were determined using the bicinchoninic acid (BCA) method (18) according to the manufacturers' instructions.

1.3 RESULTS AND DISCUSSION

Results

Microsomes prepared from sheep liver tissue by PEG precipitation yielded the highest protein levels of and a three fold increase in the yield was obtained compared to the yield obtained with CaCl_2 precipitation (table 3.1).

Table 3.1: Comparison of protein and cytochrome b_5 in microsomes prepared by CaCl_2 and PEG precipitation.

	Fresh liver (g)	Final Volume (ml)	Protein Concentration (mg/ml)	Total protein (mg)	Total cytochrome b_5 isolated (μmol)
PEG	350	250	32.48	8120.00	1.64
CaCl_2	350	220	18.41	4050.20	0.56

Microsomes were solubilised with Triton X-100 for the isolation and purification of cytochrome b_5 . The resulting solubilised microsomal solution was centrifuged to obtain a clear red supernatant which was subjected to ion-exchange chromatography. A gradient system was developed for the chromatographic separation by combining the most advantageous aspects of the two methods described by Strittmatter *et al.* (11) and Yang and Cederbaum (10). It was found that after a second round of anion exchange chromatography, no further purification took place with either ion exchange or size exclusion chromatography (tables 2 and 3).

The purity of both batches of cytochrome b_5 , isolated by the two different methods, was assessed by means of SDS PAGE analyses (figure 3.1). Protein bands corresponding to two distinct molecular mass species, a higher molecular mass specie of approximately 60,000 Da and one with a lower molecular mass of approximately 16,000 Da were observed, representative of the tetrameric and monomeric forms of ovine liver cytochrome b_5 respectively.

Table1: Summary of purification procedures in cytochrome b_5 isolation from CaCl_2 precipitated sheep liver microsomes.

Fraction	Total volume (ml)	[Protein] (mg/ml)	Cytochrome b_5 (μM)	Total cytochrome b_5 (μmol)	Specific activity ($\mu\text{mol}/\text{mg}$)	Purification (fold)	Yield (%)
Microsomes	100	18.4					
Triton X-100 extract	75.0	3.40	8.50	0.638	0.00250	1.00	100
1st DEAE eluate	120	0.35	4.37	0.524	0.0125	4.98	82.2

2nd DEAE eluate	28.5	0.41	8.96	0.255	0.0218	8.67	40.1
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Table2: Summary of purification procedures in cytochrome b₅ isolation from PEG precipitated sheep liver microsomes.

Fraction	Total volume (ml)	[Protein] (mg/ml)	Cytochrome b ₅ (μM)	Total cytochrome b ₅ (μmol)	Specific activity (μmol/mg)	Purification (fold)	Yield (%)
Microsomes	50.0	32.5					
Triton X-100 extract	430	3.42	1.76	0.757	0.000515	1.00	100
1st DEAE eluate	170	2.52	3.89	0.661	0.00154	3.00	87.6
2nd DEAE eluate	30.0	0.58	10.9	0.327	0.0188	36.6	43.5

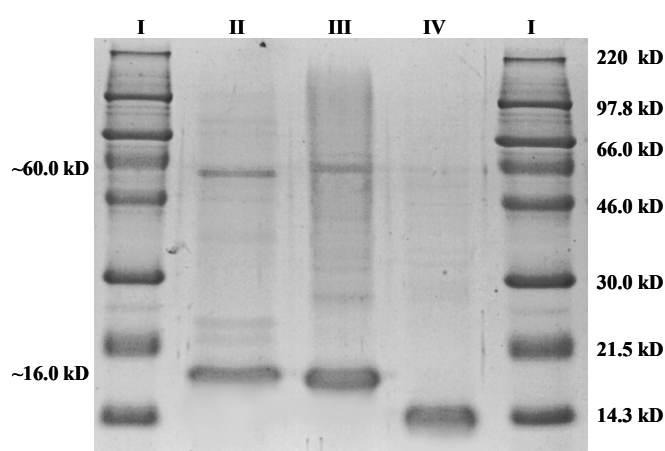


Figure 3.1 SDS PAGE analyses of purified cytochrome b₅ using a 15% resolving gel. Lane I: Molecular weight markers; lane II: Cytochrome b₅ prepared from the CaCl₂ precipitated microsomes; lane III: Cytochrome b₅ prepared from the PEG precipitated microsomes; and lane IV: Tryptic solubilised cytochrome b₅.

The hydrophobic membrane binding domain was removed by means of restricted tryptic digestion to prepare truncated cytochrome b₅. A method was developed that produced solubilised cytochrome b₅ with consistently high yields, irrespective of the source and type of trypsin used. Truncated ovine cytochrome b₅ was prepared using two types of trypsin – a twice crystallised bovine pancreatic trypsin with a high specific activity, and a low specific activity form of trypsin, which contained a high percentage of lactose. Both these enzyme preparations yielded the same amount of active truncated cytochrome b₅.

The effect of time on the exposure of cytochrome b₅ to trypsin shows that the solubilisation of approximately 0.5 ml (290 μg) purified intact cytochrome b₅ at 4 °C was complete after approximately 180 minutes. A second experiment was carried out, extending trypsin exposure to 24 hours under the same conditions. The prolonged exposure to trypsin did not alter the yield of truncated cytochrome b₅ markedly (table 4).

Table3: Analysis of the effect of trypsin and time on the purification of soluble cytochrome b_5 from intact insoluble cytochrome b_5 .

Fraction	Total volume (ml)	[Protein] (mg/ml)	Cytochrome b_5 (μ M)	Cytochrome b_5 (μ mol)	Specific activity (μ mol/mg)	Purification (fold)	Yield (%)
Intact protein	4.0	0.41	9.0	0.036	0.022		100
Truncated protein (3 hr)	4.5	0.38	5.7	0.026	0.015	0.68	72
Truncated protein (24 hr)	4.5	0.37	5.1	0.023	0.014	0.64	64

When analysed by SDS PAGE (figure 3.1, lane IV), the truncated cytochrome b_5 exhibited an apparent molecular mass of 10,000 Da while the intact form of the protein exhibited an apparent molecular mass of 16,000 Da.

Samples of the native and truncated cytochrome b_5 were subsequently analysed by ESMS (figure 3.2). Intact cytochrome b_5 exhibited a molecular mass of 15,263 Da when analysed on a quadropole ESMS (figure 3.2A). The truncated sample showed two species with different molecular masses of 9,816 Da and 10,182 Da (figure 3.2B). Both these species were present in the same ratio in the samples digested for 3 and 24 hours. A fragmentation study of each of the protein species was subsequently carried out on the quadropole ESMS. The study indicated that the two truncated species were derived from the same protein. The 10,182 Da specie correlated with the mass obtained for the truncated form of ovine cytochrome b_5 prepared according to the modified method of Omura and Takesue (19) previously used in our laboratory (20). Cytochrome b_5 truncated by TPCK trypsin immobilised on Sepharose beads yielded a protein with a molecular mass of 10,182 Da which was in accordance with results obtained by Roos (20).

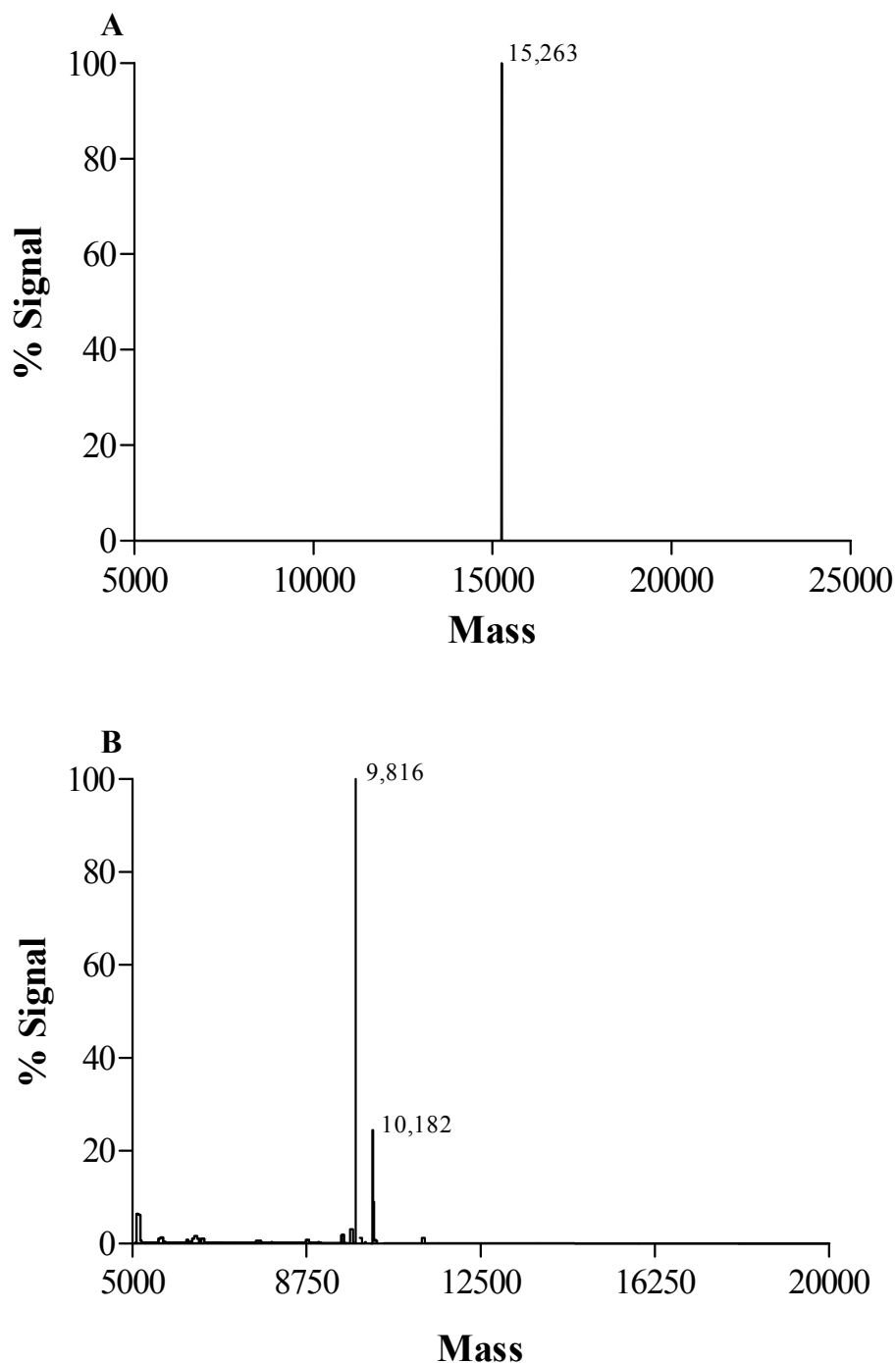


Figure 3.2 Resolved ESMS spectra of monomeric cytochrome b_5 . A: The molecular mass of intact cytochrome b_5 without its heme group. B: Truncated cytochrome b_5 showing two molecular mass species.

The proteins were subsequently analysed on a Quadrupole-Time of Flight Mass Spectrometer (Q-ToF MS) to determine the accurate masses of the different forms of cytochrome b_5 (figures 3.3 and 3.4). The molecular mass of apo cytochrome b_5 (without the heme group) and holo cytochrome b_5 (with the heme group) was 15,249 Da and 15,865 Da, respectively (figure

3.3). A sample of the trypsin truncated protein was analysed by Q-ToF MS. The analyses showed four fragments (figure 3.4A). In addition to the 9,812 Da and 10,184 Da fragments previously detected by ESMS, which differed by 370 Da from each other, two new fragments were detected at 10,028 Da and 10,399 Da, also differing by 370 Da. Non-denaturing conditions on the Q-ToF MS revealed molecular masses of only two larger species of the trypsin truncated holo cytochrome b_5 (figure 3.4B).

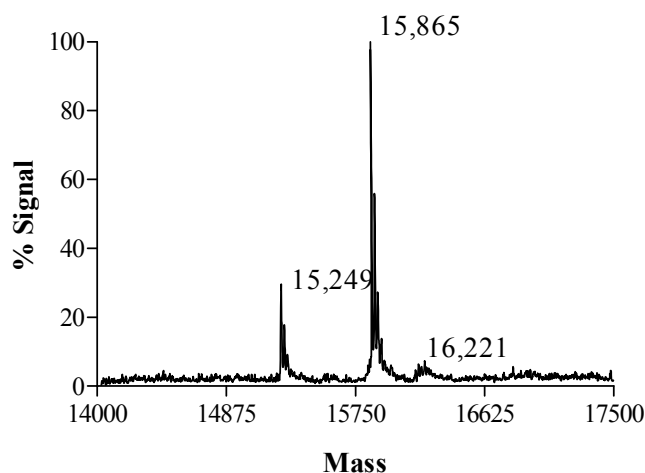


Figure 3.3 Resolved QToF MS spectrum of intact ovine liver cytochrome b_5 . The molecular mass for apo cytochrome b_5 was determined at 15,249 Da and the molecular mass for holo cytochrome b_5 at 15,865. An unidentified species with a molecular mass of 16,221 was also observed.

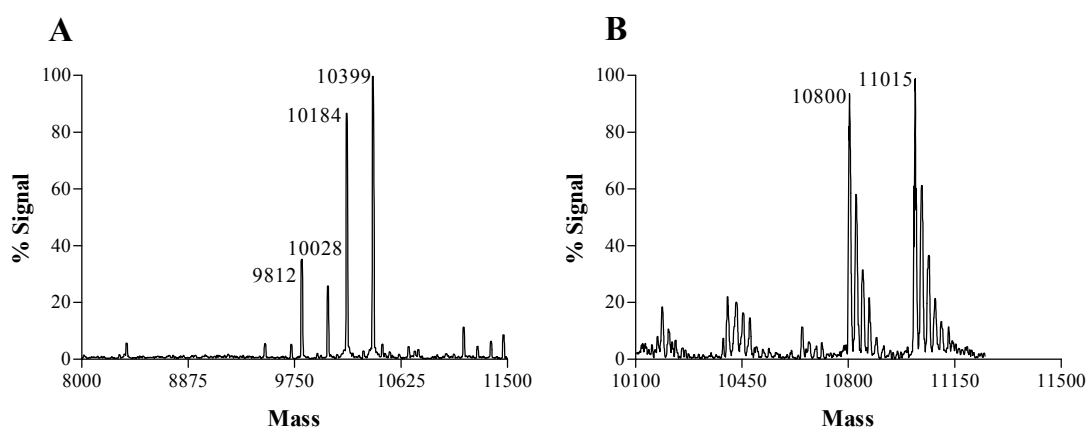


Figure 3.4 Q-ToF ESMS spectra of tryptic digested cytochrome b_5 . A: Four different species of apo cytochrome b_5 . The 10,399 and 10,028 species are most likely the products of incomplete digestion. B: Two larger species of cytochrome b_5 indicating the intact heme groups. Signals at 10822, 10844, 10866, 11027, 11049 and 11071 represent sodium adducts of the two species.

The results from non-denaturing PAGE indicated a single band for the intact cytochrome b_5 (figure 3.5). Protein that did not penetrate the gel is visible in the wells at the top of the gel. At higher protein concentrations, a faint band further down the gel became visible. The trypsin truncated form of cytochrome b_5 exhibited two distinct bands. This correlates with the ESMS results where two different species of truncated cytochrome b_5 were observed. With SDS PAGE analyses, no distinction could be made between these two species.

Absorption spectra were recorded for both the oxidised and reduced forms of the intact and truncated hemoproteins to determine whether the truncated cytochrome b_5 had retained its activity as an electron transport protein (figure 3.6). Samples were reduced with sodium dithionite. The spectra of the intact and truncated proteins were identical in the Soret region indicating that the heme binding region was unaffected by the trypsin treatment.

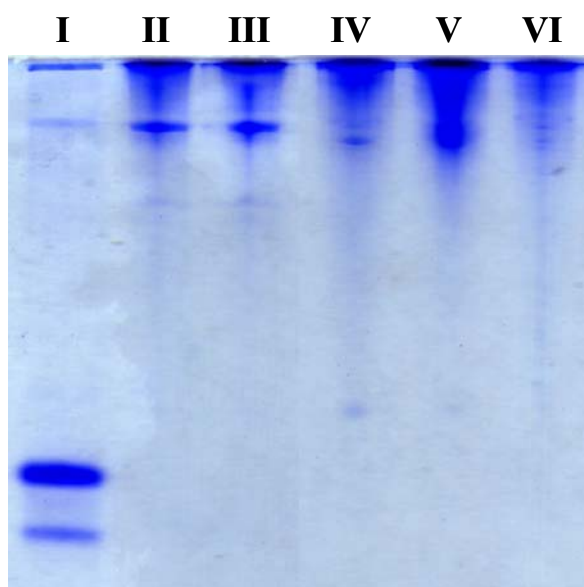


Figure 3.5 Non-denaturing gel electrophoresis of cytochrome b_5 . I: Truncated cytochrome b_5 ; II: Intact cytochrome b_5 prepared from CaCl_2 precipitated microsomes; III: Intact cytochrome b_5 prepared from PEG precipitated microsomes; IV: Sheep adrenal microsomes; V: Sheep liver microsomes and VI: Sheep testicular microsomes.

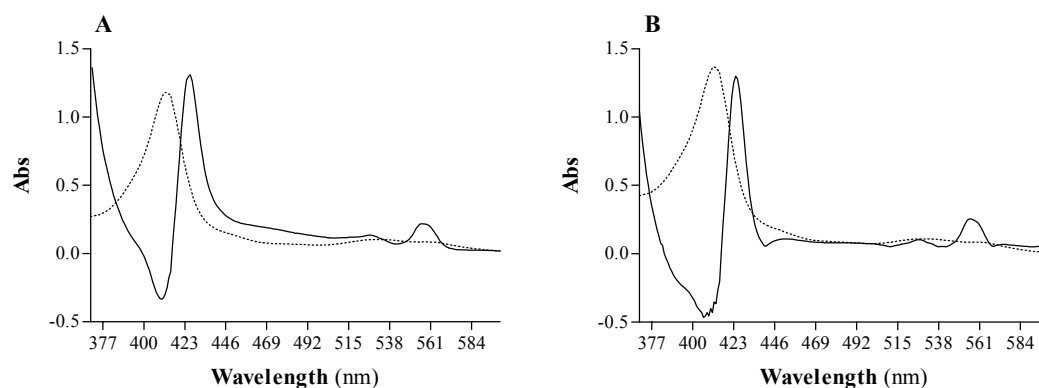


Figure 3.6 Absorption spectra of the oxidised (---) and reduced (—) forms of intact (A) and truncated (B) cytochrome b_5 . The oxidised form exhibits an absorbance maximum at 413 nm. The reduced form exhibits absorbance maxima at 556, 526 and 423 nm.

The aggregation of intact and truncated cytochrome b_5 in an aqueous environment was subsequently investigated. HPGPC analyses of intact cytochrome b_5 under conditions of low ionic strength (10 mM Tris, 0.1 mM EDTA, pH 8.15) yielded two peaks (figure 3.7A). Both fractions exhibited a typical cytochrome b_5 spectrum.

HPGPC of truncated cytochrome b_5 yielded a single peak with a retention time indicative of a molecular mass lower than any of the intact cytochrome b_5 species previously chromatographed (figure 3.7B).

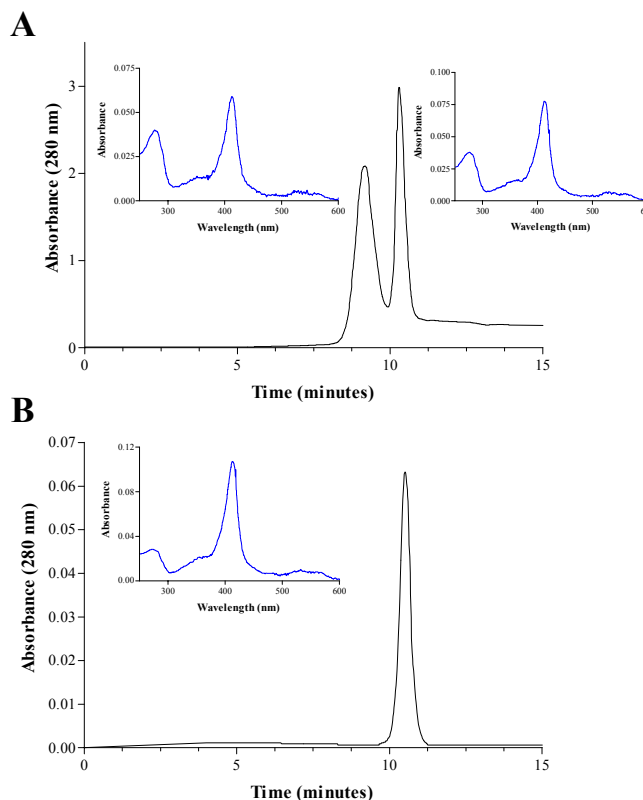


Figure 3.7 High performance gel permeation chromatograms (—) and absorbance spectra (—) of cytochrome b_5 in a low ionic strength buffer (10 mM Tris, 0.1 mM EDTA, pH 8.15). A: Two forms of cytochrome b_5 were observed at 280 nm, each with the typical cytochrome b_5 absorption spectrum; B: The truncated cytochrome b_5 sample exhibited a single peak.

Discussion

Cytochromes play a central role in mammalian metabolism and homeostasis. These hemoproteins are involved in mitochondrial electron transport and the subsequent oxidative phosphorylation of ADP, in the metabolism of xenobiotics and drug detoxification as the so-called Phase I enzymes, as well as in the biosynthesis of steroid hormones. Amongst the different cytochromes found in the mammalian body, cytochrome b_5 is the most ubiquitous hemoprotein extending the influence of the cytochromes to oxygen transport and lipid and cholesterol biosynthesis. Our laboratory is specifically interested in the role of cytochrome b_5 in ovine steroidogenesis. Most studies investigating the influence of cytochrome b_5 on mammalian steroidogenesis, utilised recombinant proteins which are modified to facilitate expression. To study the physicochemical properties of native ovine cytochrome b_5 requires a sufficient quantity of the hemoprotein.

Since published methods for cytochrome b_5 isolation from fresh mammalian tissue yielded unsatisfactory results in our hands, we modified and optimised the isolation of this protein

from sheep liver. Previously, microsomes prepared for cytochrome b_5 isolation employed ultracentrifugation for the precipitation of the final microsomal pellet. However, ultracentrifugation is not a practical option due to volume limitations and lengthy centrifugation times which are required. Strittmatter *et al.* (11) used CaCl_2 precipitation in the preparation of microsomes for subsequent isolation and purification of steer liver cytochrome b_5 . Yang and Cederbaum (10) compared PEG precipitation of liver microsomes to ultracentrifugation in the isolation and purification of rat liver cytochrome b_5 . They showed that PEG precipitated microsomes compared favourably with the microsomes prepared by conventional centrifugation. We compared PEG and CaCl_2 precipitation methods in the preparation of liver microsomes and subsequent purification of ovine cytochrome b_5 .

Our study showed that the PEG precipitation method has a number of advantages over the CaCl_2 precipitation method. No dialyses of the PEG precipitated microsomal preparation is needed before detergent solubilisation, saving at least 24 hours in the preparation of cytochrome b_5 . PEG precipitated microsomes yielded a higher protein content as well as a higher levels of cytochrome b_5 than the CaCl_2 precipitation preparation yielded (table 1).

The yields of cytochrome b_5 , obtained from the PEG and CaCl_2 methods were comparable (tables 2 and 3). SDS PAGE analyses of the purified cytochrome b_5 from the PEG and CaCl_2 precipitated microsomes indicated no difference in distribution of the monomeric and tetrameric forms. We therefore conclude that the isolation and purification method does not play an important role in the observed aggregation of the protein. ESMS was employed to verify that the 60,000 Da specie observed by SDS PAGE was indeed a tetramer of the monomeric cytochrome b_5 . Due to the nature of the operating conditions in the ESMS, heme groups are often removed from cytochrome molecules during analyses. Although a peak at m/z 615, which corresponded to the heme moiety, was observed, it could not be assumed that the molecular mass of 15,263 Da, obtained by ESMS, was the accurate mass of apo ovine liver cytochrome b_5 . ESMS analyses indicated only the monomeric form of the hemoprotein and no signal was obtained at any other m/z value up 80,000. Samples were analysed by Q-ToF MS at a low cone voltage for an accurate determination of the molecular mass. These analyses yielded mass spectra of holo and apo cytochrome b_5 . The accurate mass of both forms of sheep liver cytochrome b_5 could thus be obtained, confirming that only the monomeric form of the protein was present. All mass spectroscopic studies were carried out on cytochrome b_5 preparations obtained from both PEG and CaCl_2 precipitated microsomes.

No differences were observed. Finally the activity of both preparations was assayed using dithionite reduced vs. oxidised difference spectra. These assays confirmed that both precipitation methods yielded intact cytochrome b_5 .

It was established that cytochrome b_5 exhibits both the monomeric and aggregated forms during SDS PAGE. After being denatured and coated with SDS, samples in a SDS PAGE analyses are separated according to their mass/charge ratio, allowing for the determination of an apparent molecular mass for the different protein fractions. Samples were analysed on a non-denaturing PAGE system to obtain a qualitative indication of the presence of monomeric cytochrome b_5 under non-denaturing electrophoretic conditions. The same principles are not valid for the non-denaturing electrophoretic system where proteins are separated mainly on the basis of their charge at a given pH in the gel during electrophoresis (15). Non-denaturing PAGE analyses showed that the trypsin truncated protein has a higher net negative charge than the intact protein. It is evident that the removal of the membrane binding carboxy terminal exposed a higher number of negatively charged residues on the surface of the protein. In contrast, the full length cytochrome b_5 showed low electrophoretic mobility under non denaturing conditions. This could be ascribed to the presence of the hydrophobic membrane binding domain as well as a shielding of charges due to aggregation. Non denaturing gel electrophoresis could possibly provide the means to selectively purify a single specie of the trypsin truncated cytochrome b_5 .

Truncated cytochrome b_5 was obtained by removal of the hydrophobic membrane binding domain by restricted tryptic digestion. In our initial studies we used the method described by Omura and Takesue (19) for the preparation of truncated ovine cytochrome b_5 . Problems were, however, encountered with the commercially available trypsin. In our hands removal of the hydrophobic region of cytochrome b_5 was only moderately successful when trypsin type III or double crystallised trypsin was used. The yields were relatively low when compared to the total amount of cytochrome b_5 present in the microsomes. The average yield of solubilised cytochrome b_5 , using the method described in this study for the preparation of truncated cytochrome b_5 , was approximately 14 fold higher than yields obtained using the method reported by Omura and Takesue (19) and Roos (20) (table 4).

ESMS analyses of the trypsin treated cytochrome b_5 showed two species with different molecular masses. Both these species were present in the same ratio in both samples which had been digested for 3 hour and 24 hours thus ruling out incomplete digestion.

During the digestion, as described by Omura and Takesue (19), as well as digestion by means of immobilised TPCK trypsin, the cytochrome b_5 catalytic domain is exposed to trypsin. Immobilisation of the cytochrome b_5 on the DEAE resin protects the protein's catalytic domain. The smaller truncated specie found during immobilised digestion of cytochrome b_5 , was not observed in any of the two previously published methods used. During an investigation of rabbit cytochrome b_5 , Gibson *et al.* (21) showed that trypsin treatment of intact rabbit cytochrome b_5 yielded two distinct forms of the truncated hemoprotein with molecular masses of 9,502 Da and 10,144 Da. Previous studies indicated that the removal of the amino terminal of rabbit cytochrome b_5 by tryptic digestion, may be blocked by N-acetylation of certain amino acids (22, 23). This finding was later confirmed by Gibson *et al.* (24) when he concluded that the conversion of the third residue, glutamine, to glutamic acid was most likely due to deamination during protein isolation. In our study, using the immobilisation method for the preparation of truncated ovine cytochrome b_5 , two species with molecular masses of 9,812 Da and 10,184 Da were indicated by ESMS. Further investigation into the structure of ovine cytochrome b_5 is, however, needed to determine whether N-acetylation also occurs for sheep liver cytochrome b_5 .

It is well documented that there is a well conserved serine-lysine in the hinge region at position 90 to 91 of cytochrome b_5 . The molecular mass of this di-peptide is ~215 Da and have trypsin recognition sites on both sides of the dipeptide. It is thus highly likely that the two new species observed at 10,028 Da and 10,399 Da by Q-ToF MS analyses both have an intact serine-lysine moiety at their carboxyl termini, brought about by incomplete trypsin digestion of the protein sample. The ~370 Da difference between the species of the respective two pairs cannot be explained by any of the known sequences on the carboxyl terminal side of other species, as there are no fragments between two trypsin sites which have molecular masses of ~370 Da on either side of the serine-lysine pair (figure 3.8). This difference in size is thus most likely located in the amino terminal end of cytochrome b_5 .

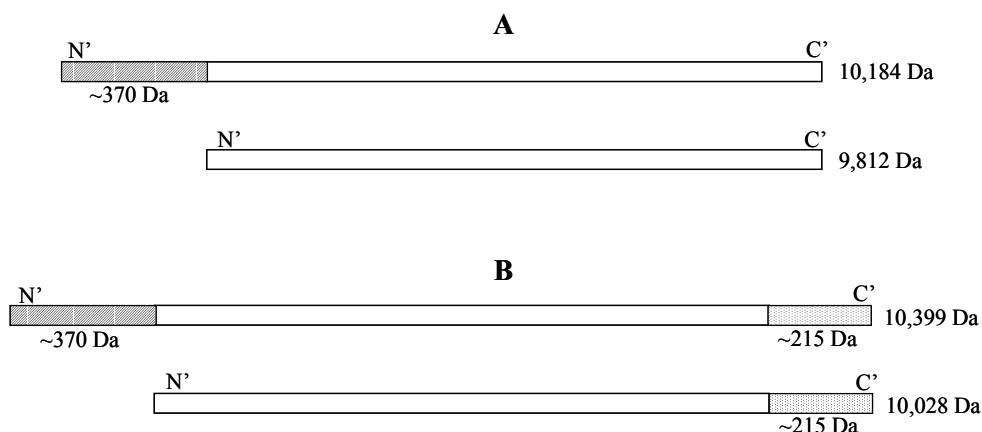


Figure 3.8 Schematic representation of the four observed species of cytochrome b_5 . **A:** Two species previously observed by ESMS with a ~ 370 Da difference in mass. **B:** Two new species observed, also with a ~ 370 Da difference, but ~ 215 Da larger than the species depicted in A.

With a significant portion of the cytochrome b_5 molecule removed by tryptic digestion, it had to be determined to what extent the smaller molecule would still be able to function as cytochrome b_5 . Difference spectra which were recorded for the oxidised and reduced forms of intact and truncated cytochrome b_5 were identical in the Soret region, suggesting that the protein domain removed by trypsin treatment might not play a direct role in the electron transfer function of ovine cytochrome b_5 .

Conclusion

It is important to achieve maximum yields when isolating proteins from small quantities of a limited source. The method reported here for the isolation and purification of cytochrome b_5 and the subsequent treatment with trypsin proved to be effective in more than one respect. The method is less time consuming than previously published methods and higher yields of intact and truncated cytochrome b_5 were obtained from the same amount of raw material. Preparing microsomes by means of ultracentrifugation would be suitable for this type of isolation, but expensive equipment is required and the procedure is extremely time consuming. Furthermore, the type of trypsin used had no influence on the yield or the integrity of truncated cytochrome b_5 .

This study showed that the use of PEG precipitation for the preparation of ovine liver microsomes has considerable advantages over ultracentrifugation in the isolation of cytochrome b_5 . A more robust, practical and reproducible method for the isolation from liver microsomes of cytochrome b_5 was achieved by PEG precipitation and modification of subsequent chromatographic steps. It is now possible to isolate cytochrome b_5 from small

samples of raw material, such as adrenal glands, in markedly shorter times with concomitant higher yields. The method is also suitable for large scale preparation in the isolation of cytochrome b_5 . In addition, a novel method for the preparation of trypsin truncated cytochrome b_5 was developed. This method resulted in considerably higher yields of truncated b_5 (14 fold) and was not dependent to the type of trypsin employed. The development of these methods will contribute markedly to the study of cytochrome b_5 .

Of all the techniques used in this study to characterise the purified intact cytochrome b_5 , it is only ESMS analyses that failed to show an aggregate form of the protein. Results obtained from the above mentioned experimental work raised the question whether cytochrome b_5 occurs as a monomer or an aggregate *in vivo*. The experiments to further investigate this question is described in Chapter 4.

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CHAPTER 4

AN INVESTIGATION OF THE AGGREGATION OF SHEEP ADRENAL CYTOCHROME b₅ *IN VIVO*

1.1 INTRODUCTION

As mentioned earlier, during the isolation and purification of ovine liver cytochrome b₅ in our laboratory, SDS PAGE analyses of the purified protein yielded two distinct bands corresponding to molecular masses of ~60,000 Da and ~16,000 Da. Even under the most stringent denaturing conditions (high mercaptoethanol levels and prolonged heat denaturation) the SDS-banding pattern of purified cytochrome b₅ did not change (1). Electrospray massspectrometry (ESMS) of the isolated cytochrome b₅ did, however, show that the protein was pure and that only one molecular specie, with a molecular mass of 15,263 Da, was present.

The ESMS data clearly indicated that native ovine cytochrome b₅ formed aggregates and that a very stable complex of tetrameric cytochrome b₅ with an apparent molecular mass of ~60,000 Da was the predominant form. The 60,000 Da complex was also identified by SDS PAGE analyses when monomeric cytochrome b₅ (Mr 15,263 Da) was isolated from SDS PAGE gels and stored at 4 °C for 2 weeks.

In the presence of high levels of detergent (deoxycholate), the monomeric specie prevailed. Other factors which are known to influence aggregation of cytochrome b₅ are the ionic strength of the solution and the protein concentration. From the results presented in the previous chapter it is clear that ovine cytochrome b₅ favours the aggregated (60,000 Da) form at physiological salt concentrations in an aqueous solution. The stable tetrameric complex of native ovine cytochrome b₅, even in the presence of the detergents used to solubilise the enzyme from the microsomal membranes, inevitably raises the question whether the native form of the protein *in vivo* is monomeric or tetrameric.

Cytochrome b₅ is an electron transfer hemoprotein that catalyses a wide range of reactions in mammalian tissue. The protein exists in two forms, an amphipathic membrane-bound form and a water-soluble cytosolic form. The membrane-bound form is found in the endoplasmic reticulum and mitochondria of all cells and consists of a hydrophobic membrane-anchoring domain and a hydrophilic catalytic domain (2). During adrenal and gonadal steroidogenesis cytochrome b₅ influences the action of the cytochromes P450-dependent steroid

17 α -hydroxylase and 21-hydroxylase (CYP17 and CYP21). These enzymes play a central role in glucocorticoid, mineralocorticoid, and androgen biosynthesis and a number of studies have shown the importance of cytochrome b₅ in C₁₉-steroid production by CYP17 (3, 4).

We have previously shown, in preliminary studies, that cytochrome b₅ influences the ratio of deoxycorticosterone to deoxycortisol production in ovine adrenal microsomes (5). During our previous investigations we encountered, what appeared to be, higher molecular mass aggregates in our cytochrome b₅ isolates. In this study the apparent aggregation of ovine cytochrome b₅ was investigated and the question arose whether the protein exists as a monomer or an aggregate *in vivo*. Previous research has shown that the aggregation of cytochrome b₅ can be abolished by detergents (deoxycholate) and it was concluded that the endoplasmic reticulum membrane will have the same influence on the enzyme *in vivo* (6). It was, however, also demonstrated by Calabro *et al.* that low protein and salt concentrations favoured smaller aggregates and that a pure monomeric form of cytochrome b₅ could be isolated by size-exclusion chromatography under these conditions (7). These results indicated that the aggregation of cytochrome b₅ *in vivo* was influenced by a number of factors.

To prevent cytochrome b₅ aggregation in aqueous solution the hydrophobic membrane-anchoring domain can be removed by means of tryptic digestion. In more recent work it was suggested that cytochrome b₅ influences CYP17 activity, not through direct electron transfer, but by steric interactions. These findings implicate that cytochrome b₅ would require its hydrophobic membrane binding domain to influence steroidogenesis (8). In this study we investigated the aggregation state of ovine cytochrome b₅ in solution, in adrenal microsomes and *in situ*.

The results of this investigation was published in Endocrine Research, Vol. 28, No. 4, pp. 489-496, 2002 (Appendix A).

1.2 MATERIALS AND METHODS

Isolation of full length and truncated cytochrome b₅

Ovine liver microsomes were prepared as described by Yang and Cederbaum (9). Cytochrome b₅ was isolated from the ovine liver microsomes as described by Strittmatter *et al.* (10).

Tryptic digestion of intact cytochrome b₅

Trypsin truncated cytochrome b₅ was prepared as described in Chapter 3.

Electrospray mass spectrometry of purified cytochrome b₅

ESMS analyses of cytochrome b₅ samples were done under conditions described by Swart *et al.* (11).

Raising of antibodies against full length and truncated cytochrome b₅

Antibodies to full length and truncated cytochrome b₅ were raised according to the method of Bellstedt *et al.* (12).

SDS PAGE and Western blot analyses

SDS PAGE was carried out according to the method of Laemmli (13). Western blot analyses were carried out with the West Dura™ western blotting kit from Pierce Chemical Co., Rockford, Ill, USA.

High Performance Gel Permeation Chromatography (HPGPC)

High performance gel permeation chromatography (HPGPC) was performed as described in Chapter 3.

Assays for adrenal microsomal steroidogenic activity

Adrenal microsomal assays were carried out as described by Swart *et al.* (5).

1.3 RESULTS AND DISCUSSION

The purity of the full length and truncated cytochrome b₅ was assessed for by means of SDS PAGE. The full-length protein exhibited a major protein band corresponding to a molecular mass of ~16,000 Da which represented the monomeric form of cytochrome b₅. Several bands corresponding to multimeric forms of the protein was, however, also observed. Electrospray massspectrometry analyses of the cytochrome b₅ solution loaded onto the gel indicated a single protein (Mr 15,260) (figure 4.1). This result confirmed that SDS PAGE normally used for the resolution of protein complexes did not fully dissociate the cytochrome b₅ aggregates.

The truncated cytochrome b_5 showed no aggregation, only a single band corresponding to a molecular mass of $\sim 10,000$ Da.

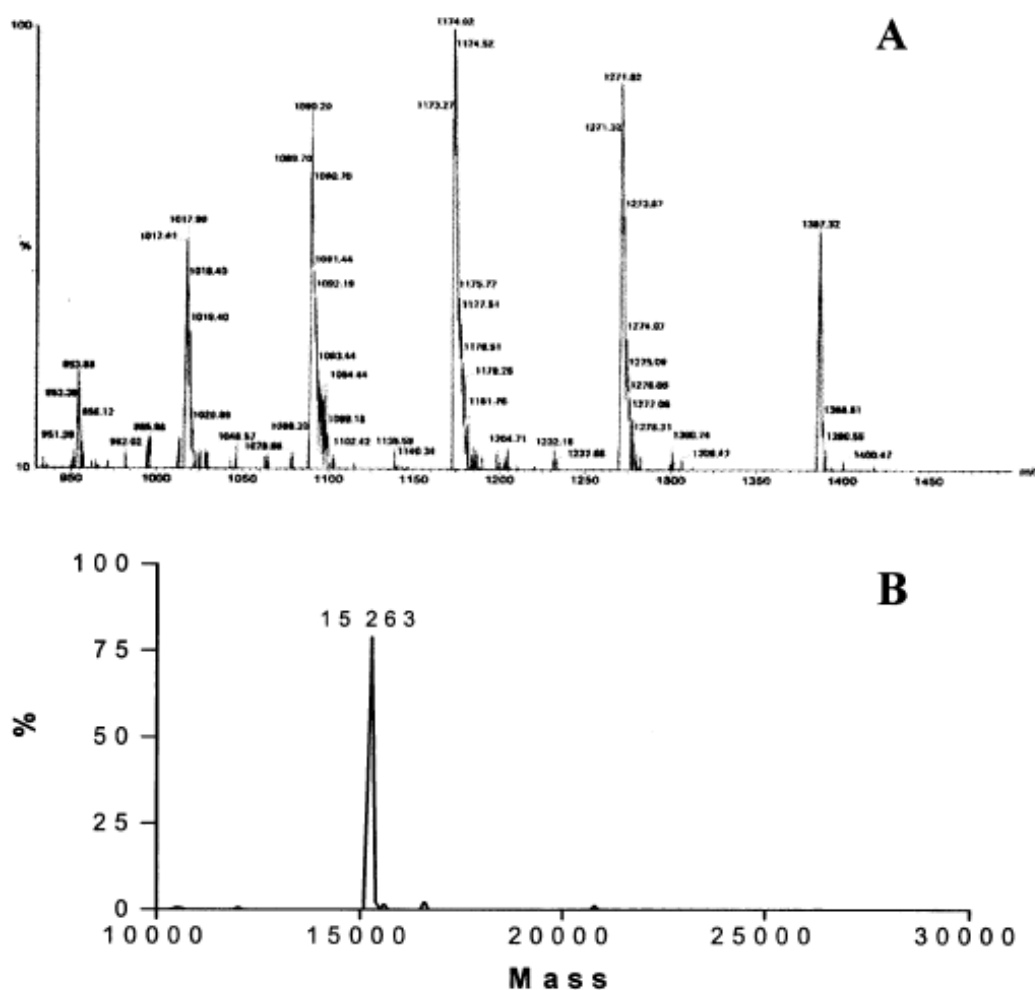


Figure 3.9 (A) Electrospray mass spectrum of purified ovine cytochrome b_5 . Each peak represents the same protein with a different charge to mass ratio. (B) Deconvolution of the ESMS spectrum was carried out by MaxEnt program 1. The molecular mass indicated for cytochrome b_5 was 15,263. No other proteins could be detected in the sample.

High performance gel permeation chromatography analyses of the purified protein under conditions of low ionic strength (10mM Tris, 0.1mM EDTA, pH 8.15) yielded two peaks (figure 4.2A). Both fractions exhibited a typical cytochrome b_5 absorption spectrum. These two fractions were collected and immediately rechromatographed under the same conditions. Each sample produced a single peak (results not shown). When the same two fractions were incubated for 24 hours before they were rechromatographed, the higher molecular mass component dissociated into two fractions while the lower molecular mass fraction remained a single peak. High performance gel permeation chromatography in phosphate buffered saline

(PBS) yielded a single peak which correlated with the higher molecular mass fraction (figure 4.2B). High performance gel permeation chromatography of truncated cytochrome b_5 yielded a single peak at a lower molecular weight than any of the other samples chromatographed. The retention time of this peak remained the same irrespective of the salt or protein concentration (figure 4.2C). These results showed that, like rabbit liver cytochrome b_5 , the aggregation of ovine cytochrome b_5 was influenced by ionic strength. In addition the aggregated form of the enzyme could be stabilized under appropriate conditions of ionic strength and pH.

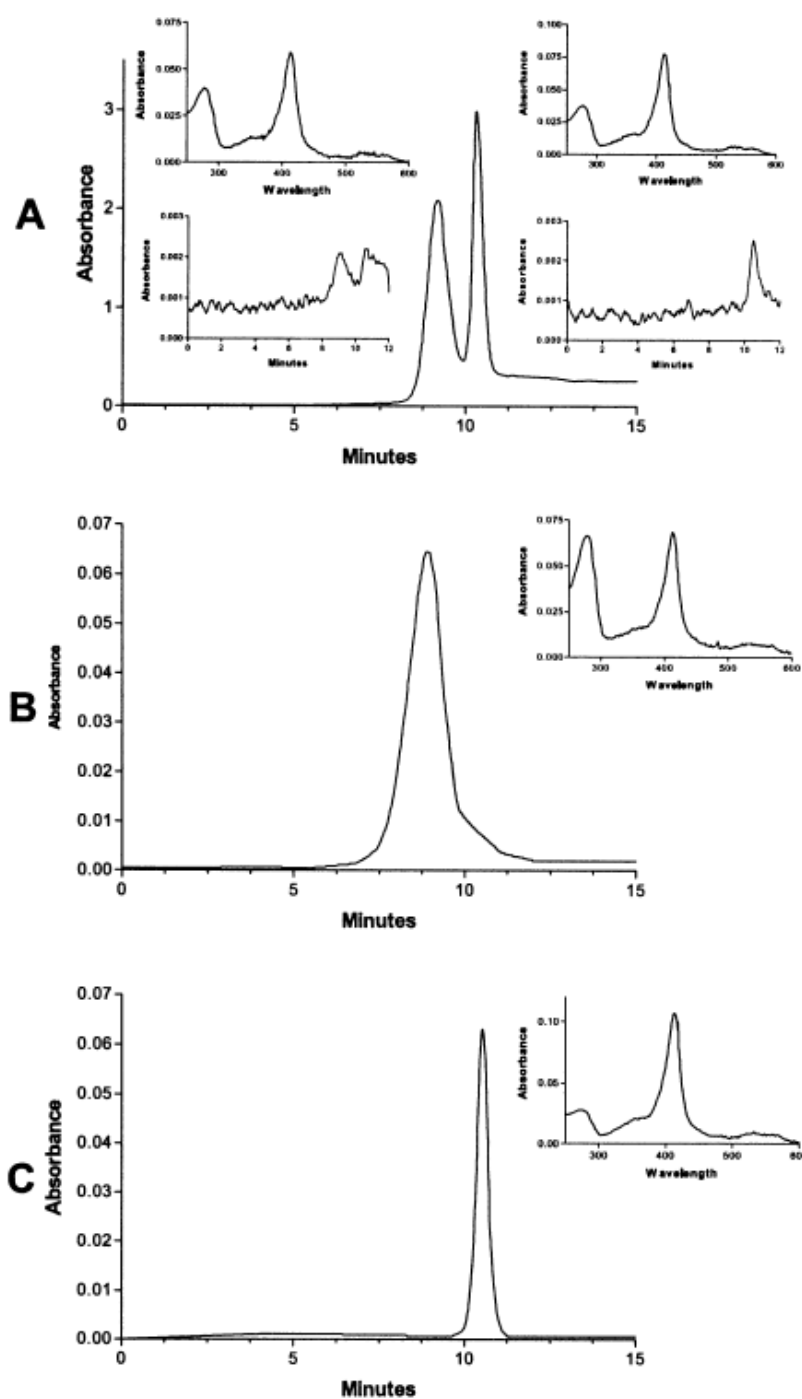


Figure 3.10 High performance gel permeation chromatogram of cytochrome b_5 in a low ionic strength buffer (10mM Tris, 0.1mM EDTA, pH 8.15) (A). Two forms of cytochrome b_5 with different retention times was observed. Both exhibited a typical cytochrome b_5 absorption spectrum. When rechromatographed after a 24-hour incubation period, the fraction correlating with the monomer still exhibited a single peak while the fraction that correlated with the aggregate again exhibited two peaks. When the buffer was changed to phosphate buffered saline, only a single peak, with the same retention time as the aggregate, was observed (B). Irrespective of the salt or protein concentration, the truncated cytochrome b_5 always exhibited a single peak (C).

The anti-serum against the intact purified cytochrome b_5 recognised the monomeric, trimeric, and tetrameric species (figure 4.3) of the enzyme. In the microsomal preparations of ovine adrenal, liver and testes and bovine adrenal the tetrameric aggregate predominated. The same anti-serum recognised cytochrome b_5 in the *zona fasciculata* of the ovine adrenal cortex as well as the adrenal medulla (results not shown). Antibodies raised against the truncated protein did not show immunoprecipitation in any of the above-mentioned microsomal preparations.

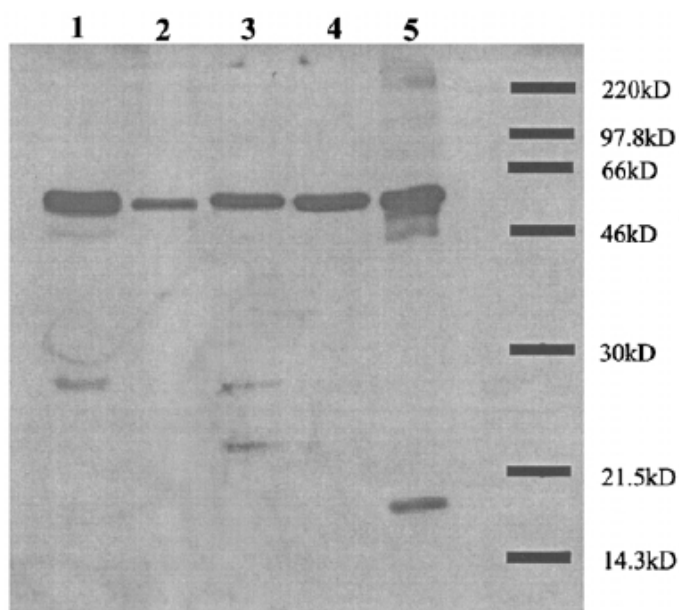


Figure 3.11 Western blot analyses of: lane 1; ovine liver microsomes, lane 2; bovine adrenal microsomes, lane 3; ovine adrenal microsomes, lane 4; ovine testicular microsomes, lane 5; purified cytochrome b_5 . Into wells 1–4, 50 mg of total protein was loaded, while 10 mg of purified cytochrome b_5 was loaded into well 5. Polyclonal rabbit anti-cytochrome b_5 was used at a dilution of 1 : 2000.

The influence of cytochrome b_5 on adrenal microsomal progesterone metabolism is shown in figure 4.4. The truncated form of the protein did not have any influence on the conversion of progesterone to deoxycortisol and deoxycorticosterone. Addition of full-length cytochrome b_5 increased the production of deoxycorticosterone with a concomitant reduction in deoxycortisol production. These results are in accordance with results previously published (5).

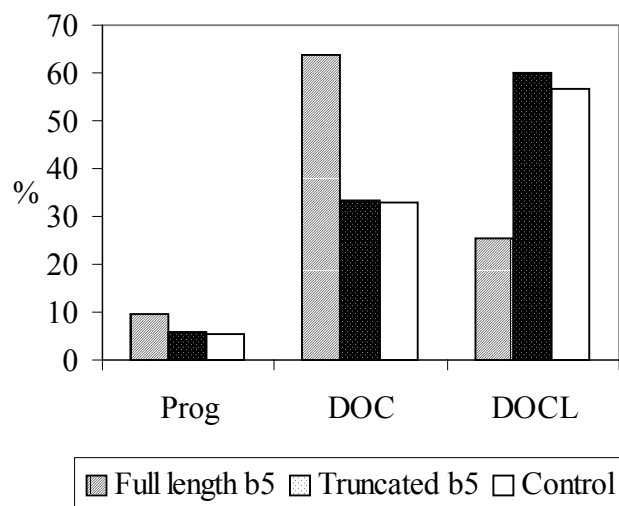


Figure 3.12 Progesterone metabolism in ovine adrenal microsomes ([cytochrome P450]=0.09 mM). Progesterone, deoxycortisol (DOC), and deoxycorticosterone (DOCL) are indicated after a 14-min incubation period. No cytochrome b₅ was added to the control which had a [Cytochrome P450] : [cytochrome b₅]=2.26. The addition of truncated cytochrome b₅ had no effect on the progesterone metabolism. Addition of full-length cytochrome b₅ (1 μ M) increased the production of DOC and concomitantly reduced DOCL production.

At physiological ionic strength and pH ovine cytochrome b₅ exists as a tetramer with a Mr of ~60,000 Da. Immunoblot analyses of ovine and bovine steroidogenic microsomes indicated the tetrameric form as the predominant specie. Although the antibody could detect the monomeric form of the purified protein no immunoprecipitation was detected at ~16,000 Da in Western blot analyses of adrenal or liver microsomes. These results indicate that under conditions of physiological pH and ionic strength the enzyme is in a tetrameric form in the endoplasmic membrane. These findings are in contrast with previously published results of Doeblner *et al.* (14) which stated that neither cytochrome b₅ nor its membrane-binding domain will self associate in phosphatidylcholine vesicles. These experiments were, however, carried out in an artificial system that contained no other proteins except cytochrome b₅. In addition, a purified recombinant mutant cytochrome b₅ was used in these studies. Although the aggregation state of intact cytochrome b₅ in adrenal microsomes cannot be readily determined, the addition of the aggregate to adrenal microsomes significantly influenced the metabolism of progesterone while the truncated form of cytochrome b₅ had no effect. The aggregation of cytochrome b₅ in the endoplasmic membrane and the influence of the further investigated.

1.4 CLOSING REMARKS

It is possible that the smallest changes in the amino acid composition of cytochrome b_5 could have a significant effect on the structure and function of the protein as was discussed in Chapter 2. A recombinant protein could thus differ only in a single amino acid from the native protein, but this substitution could have a dramatic effect on the physiological function and physical properties of the protein. We therefore investigated native cytochrome b_5 isolated from ovine liver to obtain more accurate data about the physico-chemical characteristics of this hemoprotein. The results obtained proved to yield a different perspective on these characteristics of cytochrome b_5 .

Cytochrome b_5 accepts reducing equivalents from NADH via cytochrome b_5 reductase. Both cytochrome b_5 and its reductase are bound to the endoplasmic reticulum. It has also been shown that cytochrome b_5 can be reduced by NADPH, implicating cytochrome P450-reductase as electron donor for cytochrome b_5 . The two-electron-requiring cytochrome P450 monooxygenases can receive both electrons directly from NADPH-cytochrome P450 reductase, but the second rate-limiting electron may be supplied by cytochrome b_5 . Cytochrome b_5 reductase is not obligatory for the reduction of cytochrome P450, as NADPH-cytochrome P450 reductase can receive electrons from NADPH (or NADH) and reduce either cytochrome P450 or cytochrome b_5 (figure 4.5). The difference in redox potentials of reduced cytochrome b_5 (~20 mV) and oxidised cytochrome P450 (~300 mV) dictates that electron transfer from cytochrome b_5 will be thermodynamically unfavourable. After receiving the first electron from cytochrome P450 reductase, the midpoint redox potential of the oxyferrous cytochrome P450 is sufficiently close enough (~50 mV) to that of reduced cytochrome b_5 to receive an electron from cytochrome b_5 . Should the second electron not be supplied fast enough, uncoupling could take place with a concurrent release of a superoxide anion, resulting in hydrogen peroxide formation. The suggested mechanisms of cytochrome b_5 imposed action of the cytochrome P450 monooxygenase reaction is discussed in detail in Chapter 6.

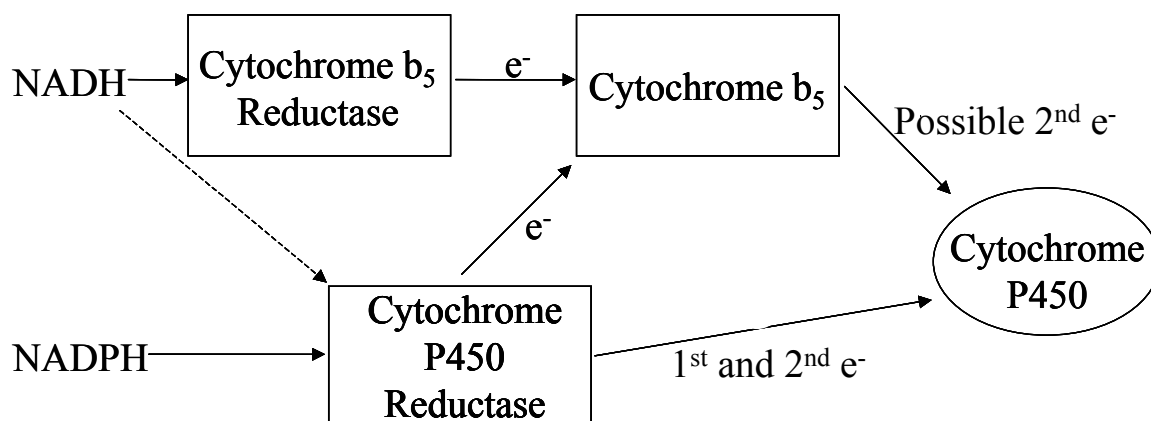


Figure 3.13 Schematic representation of electron transport from NADH and NADPH to cytochrome P450 in the endoplasmic reticulum.

Results given in Chapters 3 and 4 strongly suggest that cytochrome b₅ exists and functions as an aggregate in the microsomal membrane. The existence of an aggregated cytochrome b₅ specie in the endoplasmic reticulum would provide an effective interaction of cytochrome b₅ with either of the two reductases present and thus provide a buffer of reducing equivalents. The effect of the slow transfer of an electron from NADH to cytochrome P450 would therefore be reduced, thus minimising the chances of uncoupling taking place and producing superoxide anions. In Chapter 6 we investigate the effect of the ratio of cytochrome b₅ to cytochrome P450 on adrenal steroidogenesis.

It is currently believed that it is unlikely for a membrane-bound protein to aggregate in a phospholipid bilayer due to the hydrophobic environment. Although it is a credible argument, our results show that the detergent levels required to solubilise the microsomal membrane when extracting cytochrome b₅ could not monomerise the multimer fully. The phospholipid bilayer is a dynamic structure, allowing lateral movement of proteins embedded in the membrane. Should two or more cytochrome b₅ molecules come in close vicinity of each other inside the phospholipid bilayer, it can be expected that the molecules will attract each other with a hydrophobic force greater than that existing between cytochrome b₅ and the phospholipid membrane. HPGPC results indicate that the observed aggregation of purified cytochrome b₅ cannot be the product of the denaturing conditions of SDS PAGE. Aggregation in microsomal samples is observed beyond doubt on SDS PAGE analyses. Denaturing conditions during sample preparation, together with other membrane proteins or phospholipids, may lead to the observed aggregation. This question is addressed in Chapter 5.

Up to this point our studies focussed on the isolation and characterisation of cytochrome b₅. Cytochrome b₅ was studied as a purified protein and in microsomes. The latter can be interpreted as an *in vivo* model of the endoplasmic reticulum as all proteins present in the endoplasmic reticulum are present in the microsomes. Our results clearly show the existence of aggregated cytochrome b₅ in the microsomes.

The predominant form and mechanism of action of cytochrome b₅ in the adrenal gland *in vivo*, is still unknown. Adrenal physiology and histology are well documented and will be discussed in detail in Chapter 6. Valuable information about the function of cytochrome b₅ can be gleaned from knowledge pertaining to the localisation in the adrenal gland. Immunological studies, carried out to determine the distribution and the native form of cytochrome b₅ in the adrenal gland, will be discussed in the following chapter.

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CHAPTER 5

AN IMMUNOLOGICAL STUDY OF OVINE LIVER CYTOCHROME b₅ AND ITS AGGREGATION STATES

1.1 INTRODUCTION

Although ovine cytochrome b₅ is a monomeric protein, it is clear from the data presented in Chapters 3 and 4 that the protein also exists as a stable multimeric complex under certain conditions. In the presence of trypsin inhibitor two different forms of calf liver cytochrome b₅ were isolated by pancreatic lipase solubilisation, although trypsin digestion of these two forms yielded the same core protein (1). A larger cytochrome b₅ molecule was obtained when a detergent was used instead of lipase for the solubilisation of rabbit liver microsomes. This cytochrome b₅ molecule differed from the lipase solubilised protein by 40 amino acids, which constituted an extremely hydrophobic region in the protein (2). In an aqueous environment the cytochrome b₅, obtained from detergent solubilised microsomes, formed aggregates (3). Ito *et al.* (3) reported an octameric cytochrome b₅ specie when the protein was isolated from rabbit liver microsomes by means of detergent solubilisation. In a study using sheep liver cytochrome b₅, Lombard *et al.* (4) reported a tetrameric cytochrome b₅ specie which could be converted to the monomeric form by changing the ionic environment. Western blot analyses showed, using an antiserum against intact cytochrome b₅, positive immunostaining of a tetrameric specie only in ovine and bovine adrenal microsomal preparations. In the same study monomeric cytochrome b₅ was only detected in purified protein preparations. These results strongly suggested that, *in vivo*, sheep cytochrome b₅ exists as a tetramer, or at least as multimeric species (4).

In a previous study, carried out in our laboratory by Engelbrecht (5), anti-cytochrome b₅ rabbit IgG was obtained after immunisation with full length, intact cytochrome b₅ according to the method of Bellstedt *et al.* (6). Immunoblot analyses of purified ovine cytochrome b₅ and ovine adrenal microsomes indicated that anti-cytochrome b₅ rabbit IgG serum recognised only the cytochrome b₅ complex with a molecular mass of ~60,000 Da (figure 5.1). In the same study, no positive immunostaining occurred for the lower molecular mass (~16,000 Da) form of the protein, even though the monomeric form, prepared by preparative SDS PAGE, was used for immunisation. Similar results were obtained for ovine liver microsomes (results not shown). It thus appeared that the antibody raised against cytochrome b₅ during the

previous study, recognised only the larger molecular mass cytochrome b_5 complex and not the monomeric 16,000 Da specie.

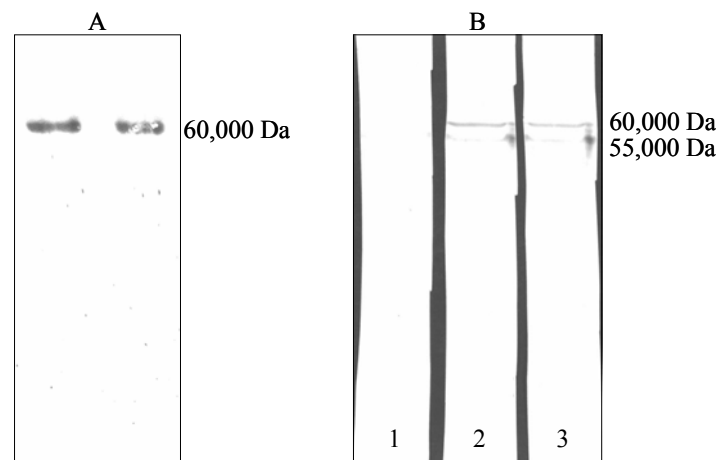


Figure 3.14 Immunoblot analyses of ovine cytochrome b_5 . (A) Cytochrome b_5 purified from ovine liver. (B) Ovine adrenal microsomes. Lane 1: Blot with day 0 serum indicating non-specific binding. Lanes 2 and 3 represent dilutions of 1:500 and 1:1,000 of the antiserum respectively. (Reproduced from 5)

The above results showed that anti cytochrome b_5 rabbit IgG serum recognised only a protein with an approximate molecular mass of 60,000 Da (tetrameric form). A faint band was detected at ~55,000 Da (not clearly visible in the image) indicative of non-specific binding. The antibody recognised the tetrameric form in the microsomal preparation. No monomer was detected. This finding posed two questions: (1) Did the antibody raised in the previous study only recognise the tetrameric aggregated form (60,000 Da) of ovine cytochrome b_5 and if this was true (2), is the tetramer of cytochrome b_5 the biologically active form of the hemoprotein *in vivo*? To shed more light on these two questions, Engelbrecht performed an immunohistochemical study using sheep adrenal tissue. This study indicated that the presence of cytochrome b_5 in the adrenal cortex was limited to the cytoplasm of the cells in the *zona fasciculata* (figure 5.2)(5). This finding was in contrast to data obtained in similar studies of the human adrenal cortex where cytochrome b_5 was indicated at high levels in the *zona reticularis* (7). In addition Engelbrecht also observed the presence of cytochrome b_5 in the nuclei of all cells in the adrenal medulla. It was concluded that, *in vivo*, cytochrome b_5 occurs as an aggregate in the sheep adrenal glands (5).

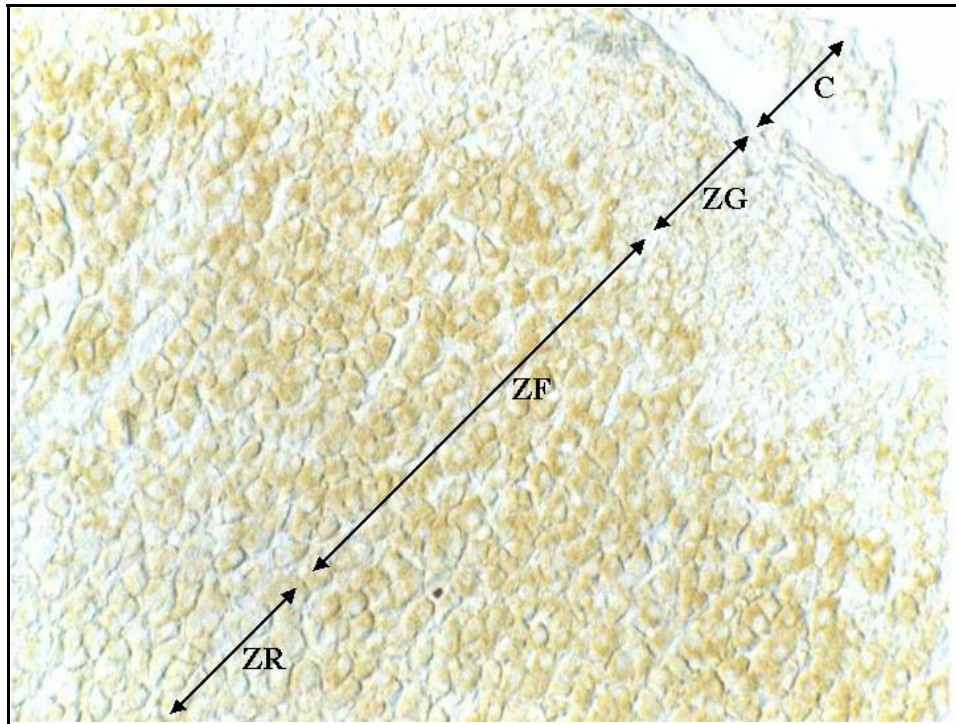


Figure 3.15 Immunohistochemical study of a cross-section of an ovine adrenal cortex showing positive staining in the cytoplasm of the cells of the *zona fasciculata* with anti-cytochrome b_5 serum (C: Capsule; ZG: *zona glomerulosa*; ZF: *zona fasciculata*; ZR: *zona reticularis*). Reproduced from (5).

We subsequently showed, with anti-cytochrome b_5 serum raised against full-length, intact cytochrome b_5 and using the more sensitive chemiluminescence Western blot detection system, that the serum does in fact recognise the tetrameric as well as the monomeric forms of ovine liver cytochrome b_5 (Chapter 4, figure 4.3). This finding showed that the monomeric form, although present, did not constitute a major proportion of the cytochrome b_5 in this preparation. The question of the aggregation state of cytochrome b_5 *in vivo* was therefore not conclusively answered. A method to discriminate between the monomeric and aggregated form of the cytochrome *in vivo* had to be developed to determine unequivocally if cytochrome b_5 does indeed exist as a tetramer *in vivo*.

The study of protein-protein interaction *in vivo* has always been a challenging one. Modern developments in fluorescent technology has led to exciting new methods, such as FRET (fluorescence resonance energy transfer), in which energy is transferred from one fluorophore-labeled protein (the donor) to another (the acceptor) with a resultant emission of light of a certain wavelength. This technology cannot, however, be effectively applied in the study of cytochrome b_5 aggregation as the so called "donor" and "acceptor" molecules are identical and it is not possible to ensure a homogenous aggregation state for cytochrome b_5 *in*

vitro. As an alternative to FRET, an immunochemical approach for the investigation of cytochrome b_5 aggregation *in vivo* was followed. A method for the separation of immunoglobulins, specifically recognising the monomeric and multimeric forms of cytochrome b_5 , was developed and these immunoglobulins were subsequently used to investigate the aggregation state of cytochrome b_5 *in vivo*.

Trypsin treated cytochrome b_5 was used to elicit an immune response in rabbits to investigate the possibility of raising a monomeric specific antibody. As an alternative, the possibility of purifying the monomer-specific antibody from the whole serum was also investigated. The preparation of an affinity chromatography matrix for the isolation of monomeric cytochrome b_5 specific antibodies was not considered, due to the aggregation of intact cytochrome b_5 in aqueous solution. The application of a nitrocellulose membrane as an affinity matrix was thus explored. Since monomeric cytochrome b_5 could effectively be separated from other protein aggregates by SDS PAGE, the separated proteins could subsequently be transferred to a nitrocellulose membrane traditionally used in Western blot analyses. The two forms of cytochrome b_5 (monomeric and tetrameric), immobilised on the nitrocellulose membrane, could subsequently be separated from one another yielding membrane strips containing either the mono or the tetrameric forms of cytochrome b_5 . The individual strips could now be used to affinity-isolate the different aggregated forms of the hemoprotein.

1.2 MATERIALS AND METHODS

Isolation of cytochrome b_5

Cytochrome b_5 was purified from fresh sheep liver as described by Swart *et al.* (8) and subjected to electrospray mass spectrometry (ESMS) as previously described (4). Truncated cytochrome b_5 was prepared by tryptic digestion from purified cytochrome b_5 as described by Lombard *et al.* (4). Purified cytochrome b_5 was analysed by SDS PAGE according to the method of Laemmli (9), using a 15% resolving gel.

Immunochemical analyses of cytochrome b_5 aggregates

Rabbits were immunised with purified truncated and full length, intact cytochrome b_5 according to the method of Bellstedt *et al.* (6). Rabbit sera containing either anti-truncated or anti-full length cytochrome b_5 immunoglobulins were used in Western blot analyses. The binding of the respective antibodies to the truncated and full length forms of cytochrome b_5 , as well as the binding to the monomeric and aggregated forms in liver microsomes was investigated. Cytochrome b_5 samples were dialysed against 10mM Tris acetate, pH 8.1,

0.1mM EDTA containing 0.25% sodium deoxycholate to prepare the monomeric form. Cytochrome b_5 predominantly in the aggregate form, were prepared by dialyses against buffer without deoxycholate. Liver microsomes, truncated and full length cytochrome b_5 was subjected to SDS PAGE using a 15% resolving gel. Two gels were prepared for Western blot analyses. The separated proteins were subsequently transferred to nitrocellulose membranes by applying a current of 1 mA/cm² membrane area for one hour in a transfer buffer (39 mM glycine, 48 mM Tris, 0.03% w/v SDS, 20% Methanol) using a horizontal semi-dry transfer system. The membranes were rinsed in PBS and reversibly stained with a Ponceau S solution (Sigma) to determine the efficiency of the transfer. The Ponceau S dye was subsequently removed from the membranes by rinsing in PBS.

The membranes were incubated in blocking buffer (154 mM NaCl, 0.5% w/v Casein, 10 mM Tris-HCl, 0.02% Thiomersal, pH 7.6) for 30 min at 25°C to minimise non-specific binding. Anti-cytochrome b_5 serum was added to the blocking buffer to a final serum dilution of 1:2000 and the membranes incubated for 90 minutes at 25°C. The membranes were subsequently washed four times for 5 min with PBS-Tween (0.1% v/v Tween 20 in PBS). Horseradish peroxidase labelled anti-rabbit antibody (Sigma) was added to the blocking buffer to a final dilution of 1:80,000 and the membranes were incubated for 60 min at 25°C. The membranes were washed as described above before adding the chemiluminescence substrate (West DuraTM, Pierce Chemical Co., Rockford, Ill, USA) and exposed to auto radiographic film.

Aggregation of purified full length cytochrome b_5 in adrenal microsomal preparations

Purified cytochrome b_5 and ovine adrenal microsomes were dialysed overnight against PBS. Cytochrome b_5 aliquots of 5 µg, 10 µg and 15 µg were subsequently added to microsomal samples (50 µg total protein) and incubated overnight at 4°C while agitating gently. Samples were subjected to SDS PAGE, using a 12.5% resolving gel. Following electrophoresis, a Western blot analyses was carried out as described above using rabbit anti-full length cytochrome b_5 serum diluted 1:2,000.

Affinity purification of immunoglobulins from rabbit anti-full length cytochrome b_5 serum

Purified cytochrome b_5 was electrophoresed using a 12.5% resolving gel, transferred to a nitrocellulose membrane and visualised using Ponceau S staining as previously described. Two horizontal strips corresponding to the bands representing the molecular weight of the tetrameric form of cytochrome b_5 at 60,000 Da (aggregate strip) and the monomeric form, at

16,000 Da (monomer strip) were excised from the membrane. The individual strips were placed in a capped 10 ml tube, washed in PBS to remove the Ponceau S and incubated in blocking buffer for 30 minutes. Anti-full length cytochrome b_5 serum was added to the blocking buffer to a final serum dilution of 1:500 and the aggregate strip was subsequently incubated for 90 minutes at 25°C while agitating gently. The immunoglobulins bound to the aggregate strip were removed from the membrane by incubating the strip in 5 ml 0.2 M glycine, pH 2.5 for 4 minutes at 25°C, while agitating gently. The solution containing the antibodies was transferred to a tube containing 0.5 ml 1 M Tris-HCl, pH 8.0 and the pH adjusted to 7. This process was repeated three times after which the monomer strip was incubated in the serum. The membrane was subjected to the same procedure as described above to isolate the immunoglobulins bound to the monomer strip. The purified immunoglobulins were stored at -20°C until used undiluted in Western blot analyses.

Immunochemical analyses of cytochrome b_5 using affinity purified immunoglobulins

Truncated cytochrome b_5 and full length cytochrome b_5 , in the monomeric and aggregated forms, were separated by SDS PAGE or non-denaturing PAGE and transferred to nitrocellulose membranes as described above. Membranes were subjected to Western blot analyses using the isolated rabbit anti-cytochrome b_5 aggregate and monomer immunoglobulin fractions. Microsomes, prepared from sheep liver, testes and adrenal tissue as previously described (8), were subjected to SDS PAGE or non-denaturing PAGE and Western blot analyses as described above to identify monomeric and aggregate forms of cytochrome b_5 *in vivo*.

1.3 RESULTS AND DISCUSSION

Results

Intact cytochrome b_5 used to prepare the truncated form of the protein, along with truncated cytochrome b_5 , was assessed by SDS PAGE analyses using conditions favouring the monomeric state of the intact, full length protein (figure 5.3). The full length and truncated forms of cytochrome b_5 both exhibited single bands at molecular masses corresponding to the monomeric form of cytochrome b_5 .

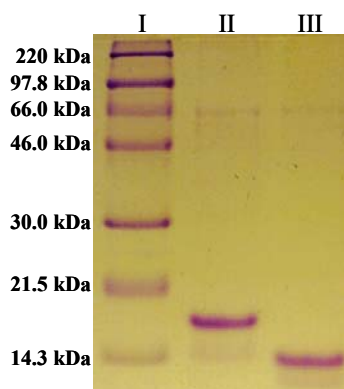


Figure 3.9 SDS PAGE analyses of purified cytochrome b_5 . lane I: molecular weight markers; lane II: intact ovine liver cytochrome b_5 (5 μ g) and lane III: truncated cytochrome b_5 (5 μ g).

ESMS analyses of the purified full-length cytochrome b_5 revealed a single specie with a molecular mass of 15,255 Da representing the protein without a heme moiety, since the heme group is detached under conditions used during ESMS analyses. Previous analyses of the different forms of purified cytochrome b_5 , carried out in our laboratory on a Micromass Quadropole-Time of Flight mass spectrometer (Q-ToF MS), identified the molecular mass of 15,249 Da for apo cytochrome b_5 (without the heme group) and 15,865 Da for holo cytochrome b_5 (with the heme group). No other protein species were detected (figure 5.4).

Q-Tof MS analyses of the truncated form of cytochrome b_5 yielded molecular masses of 9,812 Da and 10,184 Da and subsequent fragmentation studies showed that both species were derived from the same protein (4).

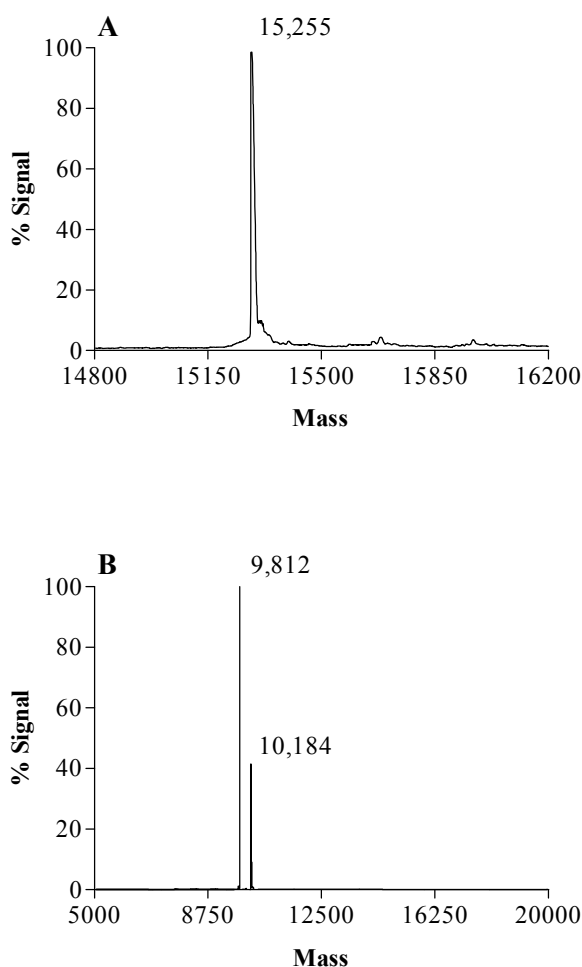


Figure 3.10 ESMS analyses of (A) purified full-length cytochrome b_5 indicating the molecular mass of apo cytochrome b_5 and (B) truncated cytochrome b_5 . No other protein species were detected, indicating the existence of a single protein in the sample.

The specificity of the antibodies in recognising the monomeric or tetrameric form of the protein was ascertained by immunostaining using rabbit sera containing either anti-truncated or anti-full length cytochrome b_5 antibodies. The rabbit anti-truncated cytochrome b_5 serum, obtained when immunising with trypsin treated cytochrome b_5 , recognised only the truncated form of the protein (figure 5.5A). The anti-full length cytochrome b_5 serum, obtained when immunising with the full length protein, recognised both the aggregate and monomeric forms of cytochrome b_5 (figure 5.5B). In addition to the monomeric and tetrameric forms observed, positive immunostaining was observed for the dimeric form ($\sim 30,000$ Da) of cytochrome b_5 . Furthermore, in liver microsomes the antibody recognised only the multimeric form of the protein (figure 5.5B).

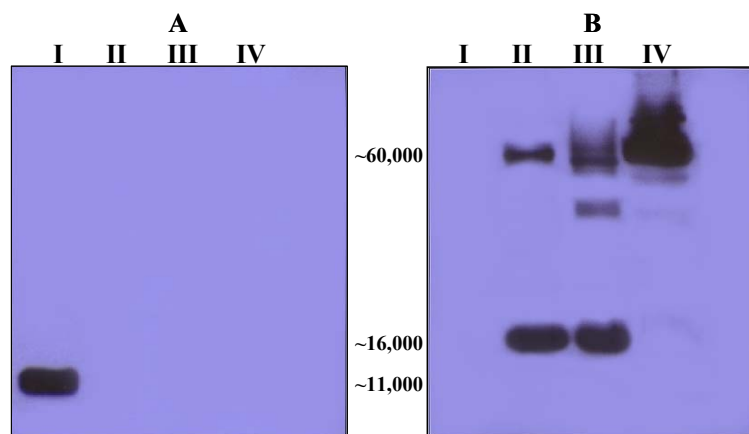


Figure 3.11 Western blot analysis of cytochrome b_5 . **A**: nitrocellulose membrane blotted with anti-truncated cytochrome b_5 serum (1:2,000) and **B**: nitrocellulose membrane blotted with anti-full length cytochrome b_5 serum (1:2,000). Lane I: truncated cytochrome b_5 ; lane II: full-length cytochrome b_5 dialysed against a deoxycholate-free buffer; lane III: full-length cytochrome b_5 dialysed against a deoxycholate containing buffer (0.25%) and lane IV: sheep liver microsomes

The possible aggregation of cytochrome b_5 was investigated in microsomal preparations to ascertain whether monomeric and/or multimeric forms of cytochrome b_5 existed in equilibrium in the endoplasmic reticulum. Increasing concentrations of full length cytochrome b_5 was added to ovine adrenal microsomes and the presence of aggregates was determined by Western blot analyses using anti-full length cytochrome b_5 serum. Although no monomeric form was detected in adrenal microsomes, only a faint band indicative of immunostaining of the monomeric form was observed at ~15,000 Da in the purified full length cytochrome b_5 preparation following dialyses with PBS (figure 5.6). Analyses clearly showed that the addition of full length cytochrome b_5 did not shift the equilibrium from the aggregated tetrameric form to the monomeric form of the protein. Although some non-specific binding was evident, no monomeric form of cytochrome b_5 was identified in adrenal microsomes. The intensity of the chemiluminescent signal corresponding to a molecular mass of 60,000 Da increased (lanes III to V) indicating that the full length cytochrome b_5 remained in the aggregated state at higher concentrations when associated with the microsomal membrane.

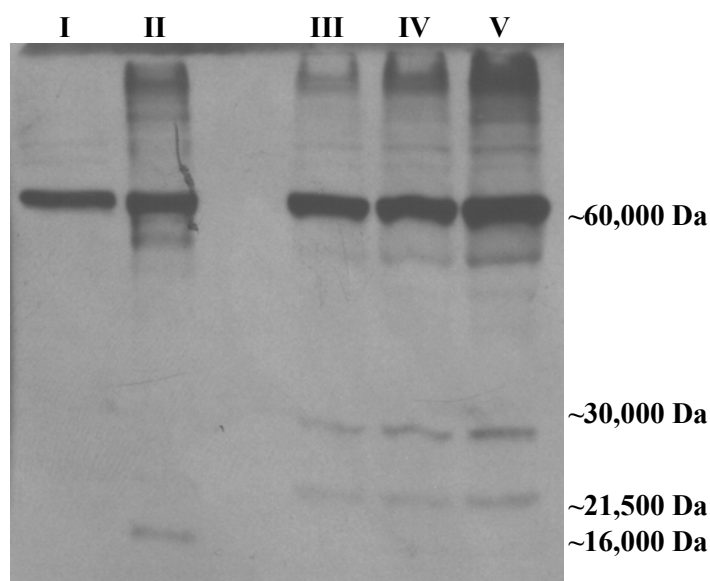


Figure 3.12 Western blot analyses of aggregation of full-length cytochrome b_5 in ovine adrenal microsomes. Lane I: adrenal microsomes (50 μ g); lane II: full-length cytochrome b_5 (5 μ g); lanes III - V: 5 μ g, 10 μ g and 15 μ g full-length cytochrome b_5 in microsomes (50 μ g). Nitrocellulose membrane blotted with antiserum against the intact form of cytochrome b_5 serum, 1:2000.

Non-denaturing gel electrophoreses was carried out with subsequent Western blot analyses using antibodies against both the purified intact and the truncated cytochrome b_5 forms (figure 5.7). Anti-truncated cytochrome b_5 serum recognised two species in the lane containing the trypsin truncated form of the protein. The antiserum against the purified intact cytochrome b_5 exhibited positive immunostaining in both the deoxycholate-containing and deoxycholate-free samples of the purified intact forms of the protein. No difference in the distribution of species were observed between the two samples of the intact protein. This is in contrast with the results obtained from Western blot analyses of cytochrome b_5 subjected to SDS PAGE, where a definite preference for the monomeric form of cytochrome b_5 was observed in the presence of deoxycholate (figure 5.3)

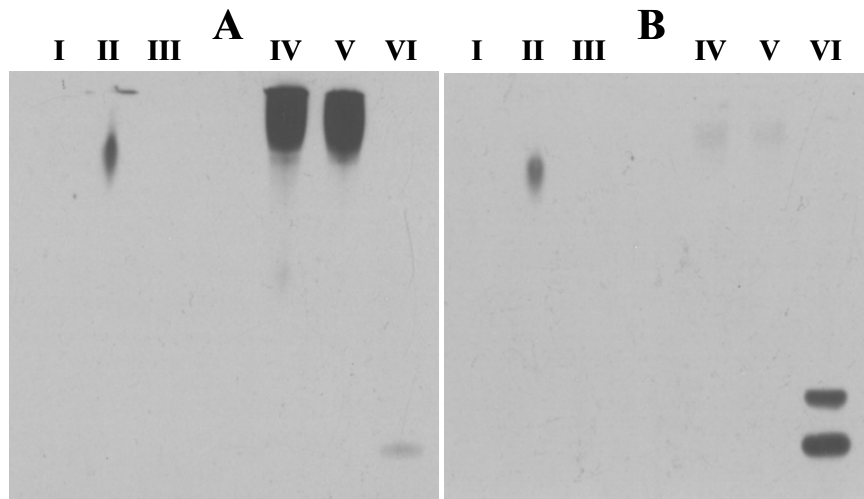


Figure 3.13 Western blot analyses of proteins separated by non-denaturing PAGE. **A**: nitrocellulose membrane blotted with anti-full length cytochrome b_5 serum and **B**: nitrocellulose membrane blotted with anti-truncated cytochrome b_5 serum. Lane I: ovine adrenal microsomes; lane II: ovine liver microsomes; Lane III: ovine testis microsomes; lane IV: full length cytochrome b_5 (PEG precipitated microsomes); Lane V: full length cytochrome b_5 (CaCl_2 precipitated microsomes); Lane VI: truncated cytochrome b_5 . A total of 50 μg protein was loaded per well. Antibody dilution 1:2000.

Since full-length cytochrome b_5 is present in different aggregate forms when not buffered in solution, the purified protein was dialysed against deionised water prior to performing electrophoresis, thus ensuring an equilibrium between the monomeric and aggregated states. The proteins were transferred onto a nitrocellulose membrane and stained reversibly with Ponceau S to visualise the monomeric and aggregate forms (figure 5.8).

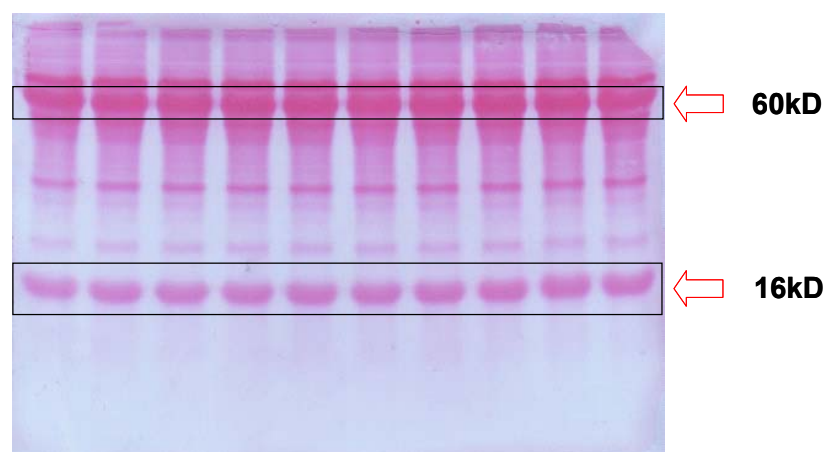


Figure 3.14 Nitrocellulose membrane with immobilised full-length cytochrome b_5 . Electrophoresed proteins, 50 μg /well, were transferred and stained with Ponceau S. The tetrameric specie (60,000 Da) and the monomeric specie (16,000 Da) are indicated.

Immunoglobulins were subsequently isolated from rabbit anti-full length cytochrome b_5 serum using aggregate and monomer strips. The specificity of these immunoglobulins was determined by ascertaining whether the purified immunoglobulins would bind the monomeric or the multimeric forms of full length cytochrome b_5 . Both the monomeric and the multimeric forms were prepared by dialysing full length cytochrome b_5 against a deoxycholate free buffer to enhance aggregation, and against a deoxycholate containing buffer to prevent aggregation. The dialysed preparations and trypsin truncated cytochrome b_5 were electrophoresed, transferred to nitrocellulose membranes and used in the analyses of the affinity-purified immunoglobulins (figure 5.9A and B). The antibodies isolated with the aggregate strip recognised the monomeric as well as multimeric forms of cytochrome b_5 . No signal was observed for the trypsin truncated form of the hemoprotein. Antibodies isolated with the monomer strip recognised only the monomeric as well as truncated forms of cytochrome b_5 . The analyses clearly shows that the presence of deoxycholate markedly increased the monomeric form of the protein.

The affinity purified antibodies were subsequently used to investigate the presence of the monomeric and multimeric forms of cytochrome b_5 in ovine adrenal, liver and testicular tissue. It is apparent that the immunoglobulins isolated with the aggregate strip recognised the tetrameric form of cytochrome b_5 as the major component of the different microsomal preparations as well as a 30,000 Da aggregate in adrenal and liver microsomes (figure 5.10A). In contrast, the immunoglobulins prepared with the monomer strip did not recognise the tetrameric form in any of the preparations (figure 5.10B).

In the adrenal and liver microsomes, the antibody against the monomeric form of cytochrome b_5 did not exhibit a significant signal for the monomeric or aggregated forms of cytochrome b_5 . In the testicular microsomes, however, clear signals corresponding to a monomer were visible at 16,000 Da and corresponding to a dimer at 30,000 Da. The identity of the proteins immunoprecipitating at a molecular mass of ~35,000 Da in the ovine testes microsomes is unknown (figure 5.10B lane III).

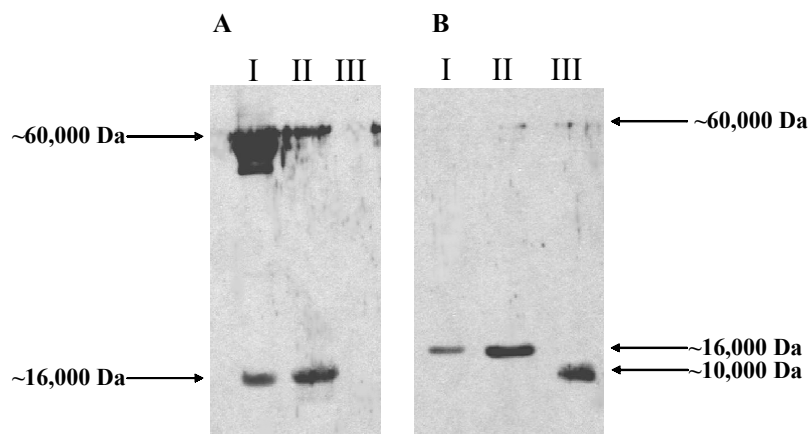


Figure 3.15 Characterisation of affinity purified rabbit anti-full length cytochrome b_5 immunoglobulins. **A:** Western blot analyses of affinity purified antibodies against the multimeric forms of cytochrome b_5 and **B:** Western blot analyses of affinity purified antibodies against the monomeric form of cytochrome b_5 . Lane I: full-length cytochrome b_5 dialysed without deoxycholate; lane II: full-length cytochrome b_5 dialysed against in deoxycholate containing buffer and lane III: trypsin truncated cytochrome b_5 . Affinity purified immunoglobulins used undiluted.

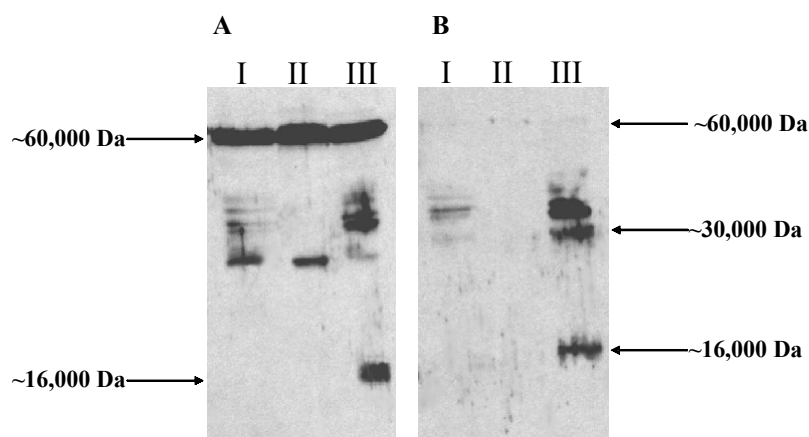


Figure 3.16 Western blot analyses of the monomeric and aggregated forms of cytochrome b_5 in microsomal tissue. **A:** nitrocellulose membrane blotted with affinity purified antibodies against multimeric cytochrome b_5 and **B:** nitrocellulose membrane blotted with affinity purified antibodies against monomeric cytochrome b_5 . Lane I: ovine adrenal microsomes; lane II: ovine liver microsomes and lane III: ovine testes microsomes. Affinity purified immunoglobulins used undiluted.

Discussion

It is evident from the data presented and current literature that cytochrome b_5 aggregation occurs. Extensive research regarding the structure of cytochrome b_5 in aqueous solutions showed that the degree of aggregation is dependent on the detergent strength and ionic conditions. However, even under conditions favouring the monomeric form of the protein viz. low salt and high detergent concentrations, a degree of aggregation was observed (4, 10). Investigation into the structure/function relationship of cytochrome b_5 and the influence of aggregation states on role of cytochrome b_5 as a redox partner would be greatly facilitated by the availability of specific antibodies recognising not only the monomeric form but also the multimeric forms. The successful preparation of a homogenous solution of monomeric full length cytochrome b_5 is hampered by the spontaneous aggregation of the protein thus impeding the preparation of cytochrome b_5 in its monomeric state for immunisation purposes. Immunisation protocols and solutions used in the preparation of the antigen favour cytochrome b_5 aggregation and therefore the host will be presented with both the monomeric and aggregate forms of cytochrome b_5 .

The truncated form of cytochrome b_5 was prepared as an antigen in an attempt to raise antibodies against the monomeric form of cytochrome b_5 only. This antiserum showed positive immunostaining with its complementary protein only. The full length cytochrome b_5 (monomer or aggregate) was not recognised by truncated cytochrome b_5 antiserum. Although the antibody against the truncated form of cytochrome b_5 could not be used to distinguish between the monomeric and aggregated forms of the protein, valuable information about the aggregation of cytochrome b_5 was gleaned from this data. Since no higher molecular mass aggregates were observed in solutions of trypsin truncated protein it is apparent that the hydrophobic membrane binding region of cytochrome b_5 is essential for the aggregation of cytochrome b_5 . In addition, the hydrophobic membrane binding domain plays an important role in the folding of cytochrome b_5 and it is clear that different protein domains are exposed as epitopes in the truncated form as opposed to those exposed in the full length native protein. This is an interesting observation since the truncated cytochrome b_5 exhibits spectral properties identical to those exhibited by the intact native protein.

Cytochrome b_5 was analysed using non-denaturing gel electrophoresis to determine the native state of protein as reported in Chapter 3. No definitive conclusions about the aggregation of cytochrome b_5 could be drawn from these results as no molecular mass markers are available

for the characterisation of proteins using non-denaturing conditions. Having raised antibodies against purified cytochrome b_5 , a method of positively identifying cytochrome b_5 was now available. The two species recognised by the anti-truncated cytochrome b_5 correlate with the species observed in the non-denaturing PAGE analyses reported in Chapter 3 figure 3.5, thus allowing the positive identification of both species as trypsin truncated cytochrome b_5 . Although SDS PAGE analyses showed a single specie, ESMS analyses results indicate the presence of two species (Chapter 3, figure 3.2B). Non-denaturing gel electrophoresis thus provide a means of separating the two trypsin truncated species of cytochrome b_5 , in the absence of denaturing agent, for subsequent elution from the gel in an active form.

The lack of difference in distribution patterns of the two intact forms of cytochrome b_5 when performing a Western blot analysis with anti-intact cytochrome b_5 shows that both the denaturing conditions of SDS PAGE and the presence of a detergent such as deoxycholate $_5$ to prevail.

Although positive staining was observed in the lanes representing the various ovine microsomal fractions on a stained non-denaturing poly-acrylamide gel (Chapter 3, figure 3.5), no positive immunostaining was observed in any of these lanes when blotted with an antiserum against full length purified cytochrome b_5 . Under non-denaturing conditions cytochrome b_5 was not released from the microsomal membrane and did not penetrate the poly-acrylamide gel. Although cytochrome b_5 is released from the microsomal membrane under the denaturing conditions of SDS PAGE the protein still exists in the aggregated form in the absence of a detergent. It may be concluded that that the conditions resulting in the release of the protein from the membrane are insufficient to prevent aggregation. This data strongly suggests that ovine cytochrome b_5 exists as an aggregate *in vivo*.

In figure 5.7 positive immunostaining was observed in the lanes representing ovine liver microsomes when blotted with both the antisera against the intact and truncated cytochrome b_5 . This observation reiterates the necessity of a pre-immune serum. Although pre-immune serum indicated no non-specific binding in Western blot analyses following protein separation on a SDS PAGE, non-specific immunostaining can still occur in Western blot analyses following protein separation on a non-denaturing gel. Proteins are denatured during sample preparation in SDS PAGE, thus destroying enzyme activity. Enzymes which could potentially compete with peroxidase for the chemiluminescent substrate would thus remain

active. In non-denaturing gel electrophoresis, certain hydrogen peroxide utilising enzymes, such as catalase, could yield false positive immunostaining.

The results presented in this chapter proves that ovine cytochrome b_5 exists in a monomeric as well as in a tetrameric form, with the tetrameric form prevailing under most conditions. The two species differ with respect to their immunogenicity and can also be distinguished immunochemically from the trypsin truncated form of the protein. These results emphasize the importance of the membrane binding carboxyl terminal of cytochrome b_5 in the maintenance of the tertiary structure of the protein as well as the formation of possible quaternary structures. We have previously shown that the cytochrome b_5 in ovine adrenal microsomes is predominantly in the tetrameric form. Other aggregation states with negligible amounts of monomeric cytochrome b_5 are also present. Addition of purified ovine cytochrome b_5 to these microsomal preparations yielded only the aggregated form of the protein.

Previous studies in our laboratory used SDS PAGE purified preparations of cytochrome b_5 , corresponding to a molecular mass of 16,000 Da for immunisation. This antiserum, however, also detected aggregate forms of the hemoprotein. Monomer- and aggregate-specific cytochrome b_5 antibodies were subsequently isolated from whole serum by transferring the cytochrome b_5 species, separated by SDS PAGE, directly from the gel onto a nitrocellulose membrane. The affinity purified immunoglobulin fraction subsequently obtained from the aggregate strip, recognised and bound both the monomeric and multimeric forms of purified cytochrome b_5 . The truncated form of the protein was not detected by these immunoglobulins. In contrast, the immunoglobulin fraction purified from the monomer strip did not recognise the higher molecular mass ($>16,000$ Da) cytochrome b_5 aggregates. Interestingly, the immunoglobulins from the monomer strip recognised the truncated form of cytochrome b_5 even though a blot with the whole serum failed to detect any truncated protein. This finding can be explained by considering the immunoglobulins present in solution — the immunoglobulin concentration was ~ 8 fold higher in the affinity purified immunoglobulin preparations than in whole antiserum. Although the concentration of immunoglobulins obtained from the monomer and aggregate strips are comparable, marked differences were shown regarding specificity of the immunoglobulins for the monomer, aggregate and truncated forms of cytochrome b_5 , again showing the pronounced influence of the membrane binding domain on the folding of full length purified cytochrome b_5 .

The affinity purified immunoglobulins were subsequently used to investigate the aggregation states of cytochrome b_5 in ovine adrenal-, liver- and testicular tissue. The immunoglobulins obtained from the aggregate strip clearly recognised a 60,000 Da form of ovine cytochrome b_5 in all three tissue types investigated. However, marked differences were observed in the lower aggregation states of the hemoprotein. The monomeric form of the cytochrome b_5 was observed in testicular microsomes only. The immunoglobulins isolated from the monomer strip did not recognise the tetrameric (60,000 Da) form of cytochrome b_5 in any of the tissue types investigated. This result clearly shows that the antigenic properties, and therefore the three dimensional structure of the monomeric and tetrameric forms of cytochrome b_5 differ substantially. It can be deduced that, *in vivo*, cytochrome b_5 occurs predominantly as a tetramer and not a monomer in liver and adrenal tissue. This difference in aggregation states in microsomal samples indicates that it is unlikely that the observed aggregation is the product of SDS PAGE sample preparation, as all microsomal samples were prepared the same. Furthermore, although aggregates with molecular masses greater than 16,000 Da are also present, the affinity purified immunoglobulins indicate that it is only in testicular microsomes that cytochrome b_5 exists as a monomer. It is evident from data presented in this study that, in steroidogenic tissue, cytochrome b_5 is present in different aggregate forms. This finding presents an interesting avenue for future investigations since the predominant form and physiological role of cytochrome b_5 in steroidogenic tissue is uncertain. Cytochrome b_5 influences steroid metabolism in the testes and the adrenal in that it modulates the catalytic activity of the cytochrome P450c17 (11, 12). Cytochrome P450c17 exhibits both hydroxylase and lyase activities for steroid hormone precursors which is influenced and possibly regulated, in part, by cytochrome b_5 in these two tissue types (8, 13, 14). It is possible that the aggregation state of cytochrome b_5 may modulate the influence the protein has as a redox partner or allosteric effector on the catalytic activity of cytochrome P450c17.

Valuable information about the function of cytochrome b_5 may be gleaned from knowledge pertaining to the localisation as well as the aggregation state of the protein. Contributions towards the elucidation of the structure/function relationship of cytochrome b_5 have been made by immunochemical investigations. Our data clearly show that the distribution of the native form of the protein *in vivo* can only be assessed with aggregate specific immunoglobulins.

In adrenal and gonadal steroidogenesis cytochrome b_5 influences the activity of the cytochrome P450-dependant steroid 17α -hydroxylase (cytochrome P450c17) reaction. As an electron transport protein, cytochrome b_5 can supply the second of two electrons to the enzyme — the first electron is predominantly supplied by NADPH via cytochrome P450-reductase. The flow of electrons from NADH to cytochrome P450 via cytochrome b_5 is not as fast as the direct input of an electron from NADPH (15). Should the second electron not be supplied fast enough though, uncoupling of cytochrome P450 with its substrate can take place with the subsequent release of superoxide. Tetrameric cytochrome b_5 in the phospholipid bilayer could thus provide an immediate source of four electrons in close contact with cytochrome P450, limiting the chances of uncoupling of the reaction .

The influence of purified full length and trypsin truncated ovine cytochrome b_5 on ovine adrenal steroidogenesis was subsequently investigated.

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CHAPTER 6

INFLUENCE OF PURIFIED CYTOCHROME b_5 ON OVINE ADRENAL STEROIDOGENESIS

1.1 INTRODUCTION

Steroidogenesis refers to the biosynthesis of steroids from cholesterol, the biosynthetic source of all steroid hormones. The primary organs involved in the biosynthesis of steroids are the placenta, the gonads and the adrenal glands (1). Steroid hormones are clinical and physiological regulators of physiological processes. Five groups of steroid hormones, which are structurally similar and arise from a common series of pathways, are generally recognised according to their physiological behaviour. These groups are mineralocorticoids, glucocorticoids, estrogens, progestins and androgens. The pathways by which these hormones are produced from cholesterol were initially studied by investigating the structures of the steroids themselves. During the past forty years, studies of enzyme kinetics and of steroidal precursor-product relationships have shown that a large number of distinct enzymes are involved in the conversion of cholesterol and its esters to active steroid hormones. Most of these steroidogenic enzymes are members of the cytochrome P450-dependent group of oxidases (2).

Three of the above mentioned groups of steroid hormones are secreted by the adrenal glands – the mineralocorticoids, which act on the renal tubules to retain sodium and thus regulate the electrolytes and water concentration in mammals; glucocorticoids, which regulate the metabolism of proteins, carbohydrates and lipids and the androgens, which induce male secondary characteristics (2, 3, 4).

1.2 THE ADRENAL GLAND

Mammals possess a pair of adrenal glands, each atop a kidney. Although they are anatomically connected, the adrenal glands function independently from the kidneys. The gland consists of two components, an inner medulla derived from the neural crest and the outer cortex derived from the coelom (5) (figure 6.1).

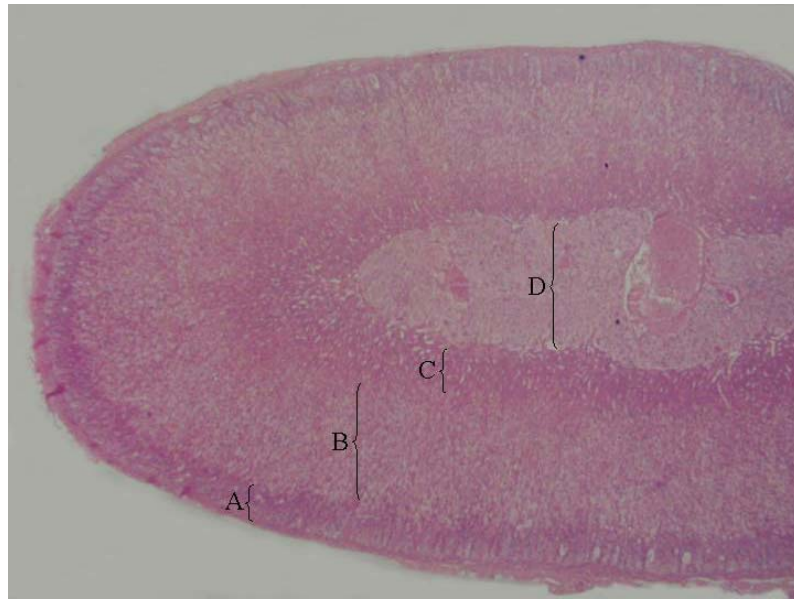


Figure 6.1 Cross-section of the adrenal gland showing the different layers in the gland. In the centre is the medulla (D), surrounded by the cortex. The cortex consists of the *zona glomerulosa* (A), the *zona fasciculata* (B) and the *zona reticularis* (C). (6)

Even though the medulla and cortex form part of the same gland, they are controlled by different systems – the medulla is controlled by the sympathetic nervous system through nerve-impulses, while the adrenal cortex is an endocrine gland. Adrenocorticotrophic hormone (ACTH) from the pituitary gland activate the adrenal cortex to release corticosteroids. Different parts of the cortex produce different hormones. The structure of the gland is discussed in more detail to gain a better understanding of the functioning of the adrenal gland.

The adrenal medulla is composed of chromaffin cells, connective tissue and numerous blood vessels and nerves. The chromaffin cells are organised in chords, surrounded by blood vessels. Features of these cells include abundant granular endoplasmic reticuli and a well-developed Golgi complex. Epinephrine and the other norepinephrine are released from 2 types of cells usually under conditions of stress and cause an increase in heart rate and blood pressure and the stimulation of the conversion of glycogen to glucose, to name but a few effects (6).

The cortex is encapsulated by connective tissue (capsule) and consists of three histologically defined zones:

- i) The outer *zona glomerulosa* is the first zone directly below the capsule and consists of small pyramidal cells arranged in rounded clusters and curved columns that are continuous

with the cords of cells in the *zona fasciculata*. The cells have a relatively small cytoplasm containing lipid droplets and abundant smooth endoplasmic reticulum. Mineralocorticoids are produced in this zone.

ii) The intermediate *zona fasciculata* consists of large polyhedral cells arranged in cords separated by sinusoids. In the cytoplasm there are numerous lipid droplets containing neutral fats, fatty acids, cholesterol and phospholipids, utilised as precursors for the steroid hormones. The cells have abundant smooth endoplasmic reticuli, mitochondria with tubular cristae and a well-developed Golgi complex. Glucocorticoids have their origin in this zone.

iii) The inner *zona reticularis* consists of rounded cells arranged in branching cords. The cytoplasm contains relatively few lipid droplets. As in the cells of the other layers, abundant smooth endoplasmic reticuli are present in these cells. Gonadocorticoids are produced in this zone, although in such insignificant small amounts, that it is not really considered a function (6).

The secretory activities of the inner two zones are controlled by the adenohypophysis through the secretion of adrenocorticotrophic hormone (ACTH). ACTH stimulates the flow of blood through the adrenal gland, promotes growth of the cortex and stimulates the secretion and release of corticosteroids. The release of ACTH is controlled through a feedback system involving the higher brain centres, the hypothalamus and the adenohypophysis (figure 6.2). Low plasma levels of corticosteroids trigger the hypothalamus to release corticotropin-releasing factor (CRF), which in turn stimulates the adenohypophysis to release ACTH. High levels of corticosteroids would inhibit CRF and ACTH release (6).

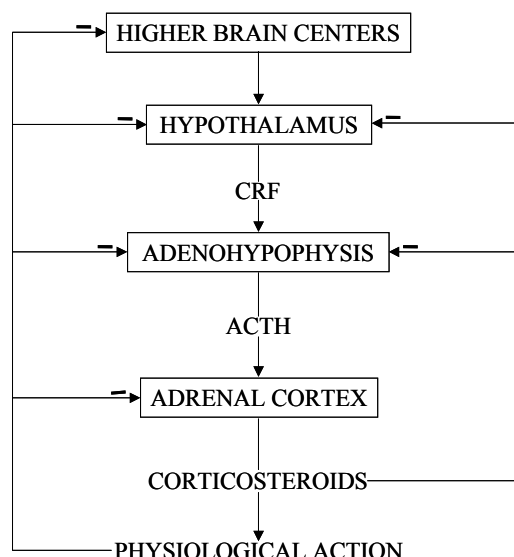


Figure 6.2 Schematic representation of neuro endocrine control of the adrenal cortex. Low levels of hormones or metabolic needs can stimulate the system. High levels of corticosteroids or the physiological action itself can inhibit the further release of hormones through negative feedback.

1.3 BIOSYNTHESIS OF ADRENAL STEROID HORMONES

Cytochrome P450 plays an important role in adrenal steroidogenesis, catalysing the biosynthesis of androgens, mineralo- and glucocorticoids from cholesterol, the steroid hormone precursor.

Although steroidogenic tissue can synthesise cholesterol *de novo*, human steroidogenic cells derive most of its cholesterol from plasma low density lipoproteins (LDL) (7,8). The rate-limiting enzyme in cholesterol synthesis is 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase. This enzyme will be suppressed if the levels of LDL in the human adrenal cells are sufficient (9). Its activity will be stimulated by the same tropic hormones that stimulate steroidogenesis, together with a stimulation of LDL receptors and uptake of LDL cholesterol. The uptake of LDL cholesterol esters takes place by means of receptor mediated endocytosis, followed by conversion to free cholesterol for immediate use in steroidogenesis or direct storage (8).

The storage of cholesterol in lipid droplets as cholesterol esters is controlled by the action of two opposing enzymes, namely cholesterol esterase and cholesterol ester synthetase. Tropic hormones such as ACTH stimulate esterase activity while inhibiting the synthetase activity, effectively increasing the availability of free cholesterol for steroidogenesis (10).

Rat steroidogenic tissue differs from human tissue in the respect that it receives most of its cholesterol from high density lipoproteins (HDL) instead of LDL. The uptake of HDL differs from LDL uptake as it is taken up through a mechanism that does not involve the internalisation of the intact lipoprotein particle (11).

Free cholesterol is insoluble in the aqueous cytosolic environment. ACTH induces a sterol carrier protein 2 (SCP-2) which transports the free cholesterol from the cytosol to the mitochondria (12,13). The flux of cholesterol over the mitochondrial membranes to the cytochrome P450_{scc} (side-chain cleavage) was thought to be stimulated by a 30-amino acid steroidogenesis activator peptide (14). This peptide has a short half-life and is very sensitive for cycloheximide. This was shown when an accumulation of cholesterol was observed in the outer membrane of the adrenal mitochondria of rats treated with cycloheximide (15). It has since then been proven that the import of cholesterol into the mitochondria is facilitated by the steroidogenic acute regulatory protein (StAR) (16).

Cholesterol is converted to pregnenolone by cytochrome P450_{scc} in the mitochondria, which is accomplished in three sequential reactions on a single active site. Cholesterol undergoes 20-hydroxylation, 22-hydroxylation and finally scission of the 20,22 C-C bond to yield pregnenolone and isocaproaldehyde (figure 6.3) (2). Each of these reactions requires a pair of electrons. The StAR-P450_{scc} system is rate-limiting and hormonally regulated and it can thus be said that it is the quantitative regulator of steroidogenesis (17).

The pregnenolone that is formed subsequently diffuses to the endoplasmic reticulum. If cytochrome P450_{c17} is not present, like in the *zona glomerulosa*, C-21 17-deoxysteroids (e.g. Aldosterone) products are formed. C-21 17-hydroxysteroids are formed (e.g. cortisol) when just the 17 α -hydroxylation activity is present. In the presence of both the hydroxylation and lyase activity, C-19 precursors of the sex steroids are formed (figure 6.3). Although cytochrome P450_{c17} is not the only cytochrome P450 that can catalyse more than one reaction, it is to date the only one described in which these activities are regulated differentially by a physiological process. Cytochrome P450_{c17} can therefore be seen as the qualitative regulator of steroidogenesis, as it determines which steroids are going to be synthesised (2, 3, 17).

The relative activities of 3 β -hydroxysteroid dehydrogenase-D⁵-isomerase (3 β HSD) and cytochrome P450_{c17}, will determine pregnenolone metabolites (figure 6.3). Pregnenolone

will be preferentially oxidised and isomerized to progesterone when just the 17α -hydroxylation activity is present, which in turn will be hydroxylated at the C-21 position to yield 11-deoxycorticosterone if the 21-hydroxylase activity is higher than that of the 17α -hydroxylation activity. In contrast pregnenolone can undergo 17α -hydroxylation to yield 17-hydroxypregnenolone and be converted to dehydroepiandrosterone (DHEA), providing that the lyase activity is higher than the 3β HSD activity. Likewise, progesterone can undergo 17α -hydroxylation to form 17-hydroxyprogesterone which in turn can, under the influence of $17,20$ -lyase activity, be converted to androstenedione. Through 3β HSD activity 17-hydroxypregnenolone and DHEA can be converted to 17-hydroxyprogesterone and androstenedione respectively. 17-Hydroxyprogesterone can be metabolised by means of hydroxylation of the C-21 by cytochrome P450c21 to 11-deoxycortisol (2,3,17).

Both 11-deoxycorticosterone and 11-deoxycortisol are transferred back to the mitochondria where conversion to corticosterone and cortisol, respectively, by the 11β -hydroxylation activity of cytochrome P450 11β takes place. 11-Deoxycorticosterone can be converted to aldosterone by the 11β -hydroxylase, 18-hydroxylase and 18-oxidase activity of aldosterone synthase (2,3).

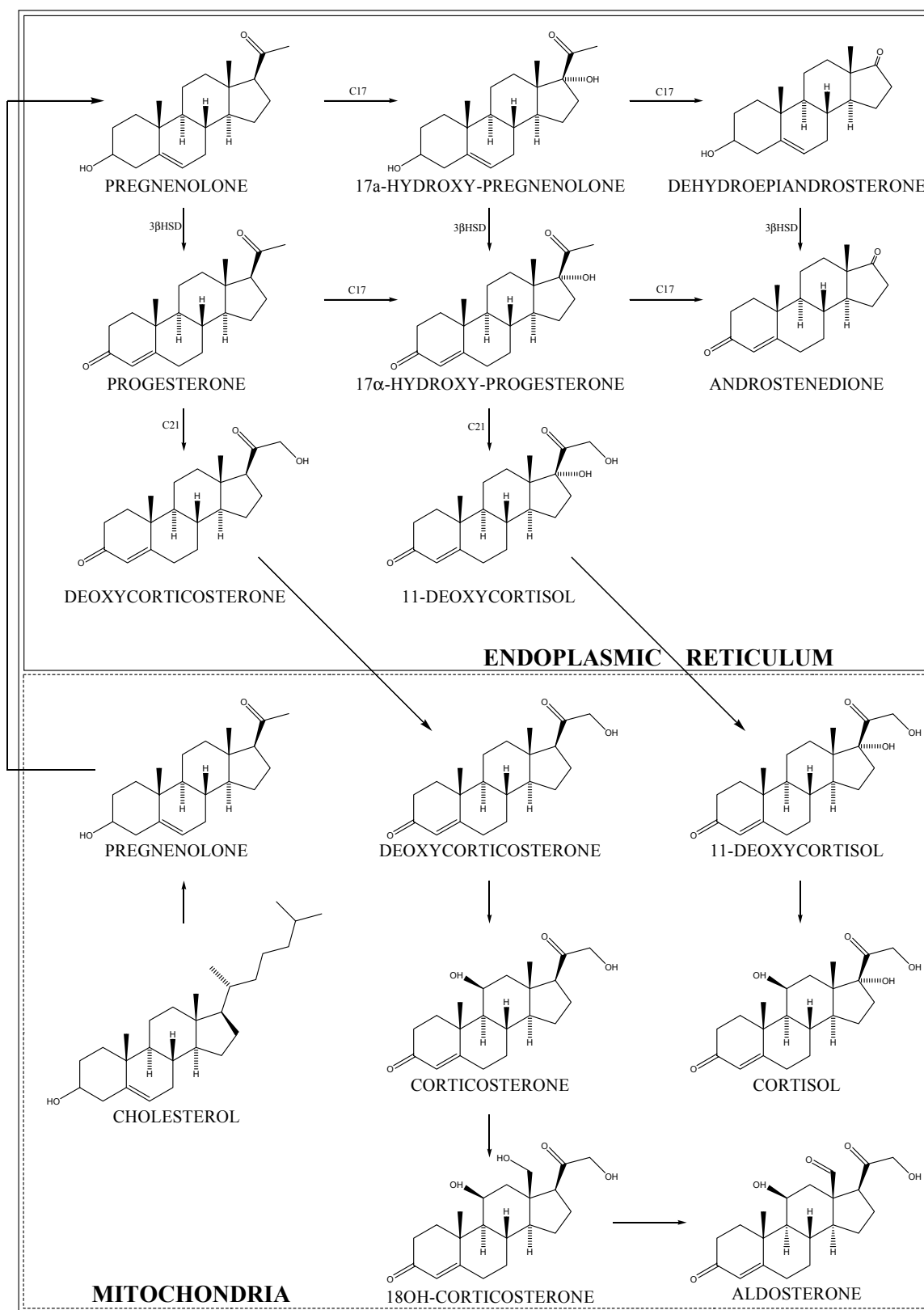


Figure 6.3 Schematic representation of adrenal steroidogenesis. The dotted line indicate the mitochondrial membrane while the inner solid line indicates the endoplasmic reticulum membrane. C17: cytochrome P450c17; 3βHSD: 3β-hydroxysteroid dehydrogenase-D⁵-isomerase; C21: cytochrome P450c21 (3)

1.4 CYTOCHROMES P450

The term “Cytochrome P450” refers to a group of heme containing proteins that have a sulphur atom ligated to the heme iron and that, upon reduction, form carbon monoxide complexes with a characteristic absorption maximum at 450nm (18, 19) (figure 6.4). Cytochrome P450s form part of a group of enzymes that insert one atom of atmospheric oxygen into their substrate and the other one into water, termed monooxygenases. Cytochrome P450-dependent monooxygenase systems are widely distributed in animals, plants and protists and catalyse a wide range of reactions. The substrates for some of these reactions include steroids, eicosanoids, fatty acids, lipid hydroperoxides, retinoids, acetone and xenobiotics (18, 20, 21).

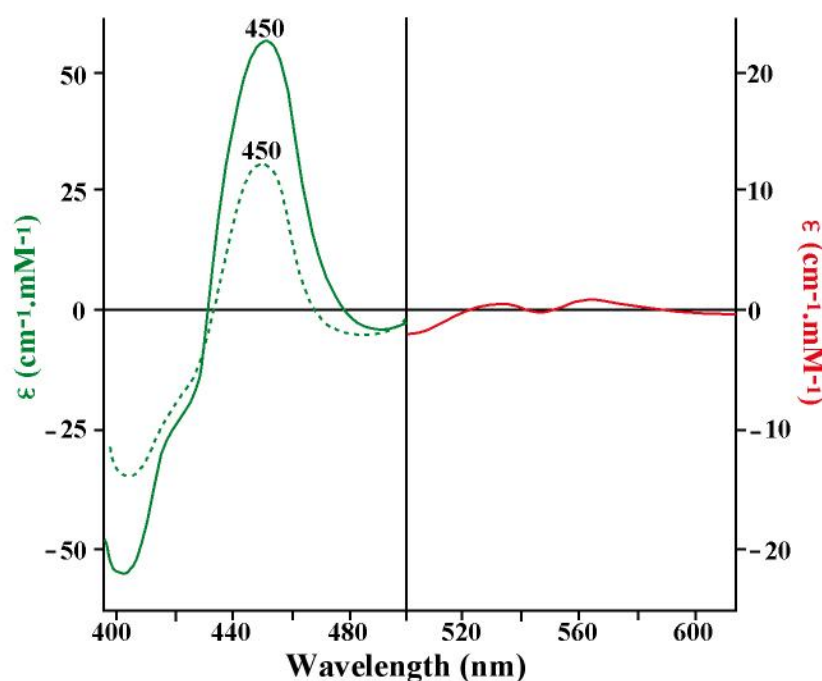


Figure 6.4 Carbon monoxide difference spectra of rat liver microsomes. The solid line represents the spectrum for carbon monoxide with diphosphopyridine nucleotide (DPNH) reduction. The dashed line represents the spectrum for carbon monoxide with dithionite reduction (Redrawn 22).

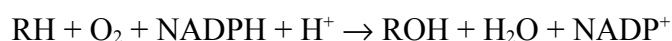
1.5 CATALYTIC CYCLE OF CYTOCHROME P450-DEPENDENT HYDROXYLATIONS

Cytochrome P450s all reduce atmospheric O_2 with electrons originating from NADPH. Cytochrome P450c17, for instance, receives electrons from a membrane-bound flavoprotein, P450 reductase. P450 reductase is in turn reduced by two electrons from NADPH. Electron

transfer from P450 reductase to cytochrome P450 is sequential (one electron at a time). The transfer of the first electron is fast while the transfer of the second one is slower (2, 23). This second electron may also be provided by cytochrome b_5 .

The catalytic reaction cycle of cytochrome P450 occurs in a number of stepwise reactions (19) (figure 6.5):

1. the binding of the substrate;
2. one-electron reduction of the ferric enzyme-substrate complex; this reducing equivalent is supplied by NADPH to the mitochondrial and the microsomal cytochrome P450s;
3. binding of molecular oxygen to the ferrous enzyme-substrate complex; superoxide can be produced at this step;
4. transfer of the second electron from either cytochrome P450-reductase or from cytochrome b_5 , the oxygen complex may dissociate to ferric cytochrome P450 and superoxide if the electron transfer is slow (mentioned in previous step);
5. protonation of the distal oxygen atom by a hydrogen atom. The production of hydrogen peroxide at this step is possible;
6. cleavage of the oxygen-oxygen bond to yield a reactive iron-oxo intermediate;
7. insertion of the activated oxygen atom into a carbon-hydrogen bond of the substrate, and
8. dissociation of the product and the regeneration of the ferric cytochrome P450.



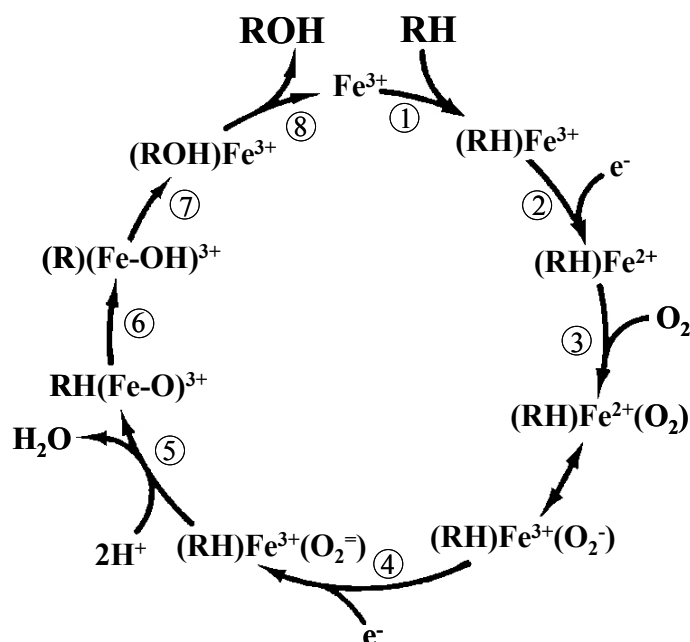


Figure 6.5 Proposed scheme for the mechanism of action of cytochrome P450 in hydroxylation reactions. RH represents a substrate and ROH the corresponding product (19).

1.6 ELECTRON TRANSPORT TO CYTOCHROME P450

6.6.1 Electron transfer in mitochondria

Cytochrome P450_{scc} functions as the terminal oxidase in the mitochondrial electron transport system. Electrons from NADPH are accepted by adrenodoxin reductase, a flavoprotein which is loosely associated with the mitochondrial inner membrane. Adrenodoxin reductase passes these electrons on to adrenodoxin, an iron-sulphur protein in solution in the mitochondrial matrix. Adrenodoxin transports the electrons to cytochrome P450_{scc} (24, 25) (figure 6.6). The adrenodoxin-adrenodoxin reductase system serves as a generic electron transport system for all cytochrome P450s in the mitochondria (26).

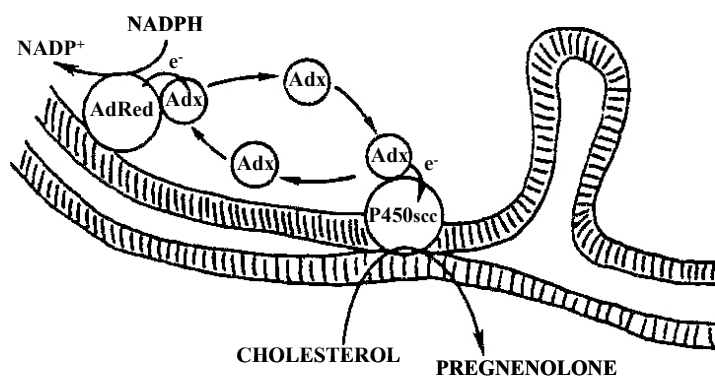


Figure 6.6 Electron transport to mitochondrial cytochromes P450 (2).

Adrenodoxin forms a 1:1 complex with adrenodoxin reductase, dissociates, and then forms a 1:1 complex with cytochrome P450_{scc}, cytochrome P450_{c11} or aldosterone synthase. The selectivity of the reaction seems to be determined by the presence of the substrate bound to the cytochrome P450 moiety (27).

6.6.2 Electron transfer in the endoplasmic reticulum

Cytochrome P450 reductase, a membrane associated flavoprotein, receives two electrons from NADPH, which is passed on to cytochrome P450_{c17} or cytochrome P450_{c21} one at a time (28, 29) (figure 6.7). The transfer of the first electron is fast and the second slower. The second electron may, however, be supplied by cytochrome *b*₅. The formation of a complex between cytochrome P450, cytochrome P450 reductase and cytochrome *b*₅ is facilitated by certain phospholipids, resulting in more efficient electron transfer. It has been reported that there are higher concentrations of cytochrome P450_{c17} and cytochrome P450_{c21} in the endoplasmic reticulum than cytochrome P450 reductase and the enzymes must therefore compete for reducing equivalents. Since phospholipids play a role in complex formation, they may also play a role in determining whether the reaction takes place at all (30, 31).

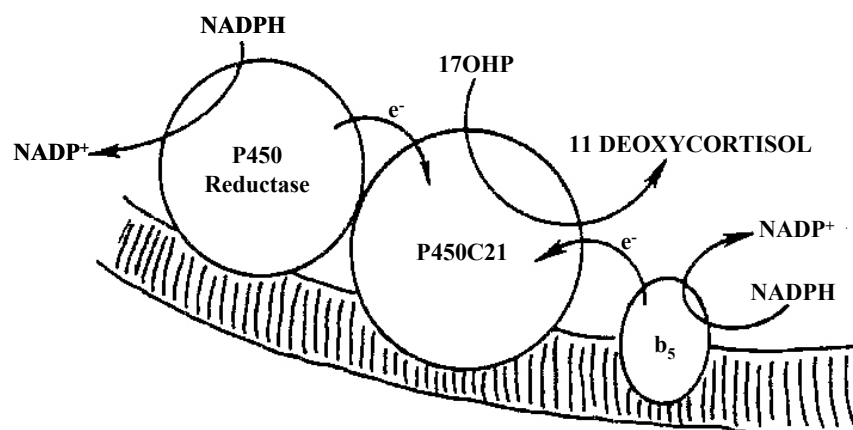


Figure 6.7 Electron transport to microsomal cytochromes P450 (2).

The availability of electrons seem to determine whether cytochrome P450_{c17} carries out only 17 α -hydroxylation, or 17,20-bond scission as well. If the ratio of reductase or cytochrome *b*₅ to cytochrome P450_{c17} increase *in vitro*, the ratio of 17,20-lyase activity to 17 α -hydroxylation increases. Competition for available substrate between the 21-hydroxylation or 17,20-lyase reaction does not seem to influence the hydroxylase and lyase reactions, as there is no significant increase in androstenedione levels if 21-hydroxylation is eliminated (32, 33).

The increase in the ratio of cytochrome P450 reductase to cytochrome P450 increased the 17,20-lyase activity in both the adrenal and the testes. The porcine testis contains 3-4 times more reductase than the porcine adrenal. This ratio seems to be the most important factor in determining whether a steroid will undergo 17,20-bond scission after 17 α -hydroxylation (33).

1.7 THE ROLE OF CYTOCHROME b_5 IN CYTOCHROME P450 CATALYSED REACTIONS

6.7.1 *Influence of cytochrome b_5 on cytochrome P450 catalysed reactions*

The involvement of cytochrome b_5 in cytochrome P450 reactions was first suggested by Hildebrandt and Estabrook (23) in 1971. Ever since, it has been a subject of dispute. Some investigators found that cytochrome b_5 had no effect on cytochrome P450 reactions, while others found that cytochrome b_5 was almost obligatory for catalysis involving certain substrates. Enzyme activity could only be shown in reconstituted systems with cytochrome P450 and cytochrome P450 reductase, which argued against the obligatory role of cytochrome b_5 . On the other hand, antibodies against cytochrome b_5 or cytochrome P450 reductase, used in a microsomal system with NADH, showed that cytochrome b_5 had an influence on the reaction mechanism (34).

Although this controversy still continues, the focus has shifted towards whether electron transfer from cytochrome b_5 to cytochrome P450 is necessary for the stimulatory effect. Cytochrome b_5 has a complex interaction with cytochrome P450, influenced by the type of cytochrome P450 involved as well as the substrate. It was shown that cytochrome b_5 can either be an obligatory component of a given reaction or a modulator of a reaction that would take place in the absence of cytochrome b_5 . Furthermore, cytochrome b_5 can have either a stimulatory or an inhibitory modulating effect dependant on the type of cytochrome P450 and the substrate involved. An absolute requirement for cytochrome b_5 has been shown for the metabolism of methoxyflurane for example (35). Other examples of such dependence are the metabolism of p-nitroanisole (36), 7-ethoxycoumarin (37), p-nitrophenetole (38), prostaglandins A_1 , E_1 , E_2 (39), arachidonate (40) and testosterone (41). In contrast, the presence of cytochrome b_5 inhibited the NADPH-supported metabolism of benzphetamine (42).

In liver microsomes, drug metabolism was stimulated by the addition of NADH implicating the cytochrome b_5 -cytochrome b_5 reductase system in these P450-mediated reactions (43). A second observation, in which cytochrome b_5 could be reduced by NADPH, indicated that cytochrome b_5 could receive electrons from cytochrome P450 reductase (44). Pandey *et al.* (45) reported that serine phosphorylation of cytochrome P450c17 enhanced the 17,20 lyase activity independent of cytochrome b_5 , while having no effect on the 17 α -hydroxylase activity. It was postulated that the enhancement of 17,20 lyase activity by phosphorylation is most probably due to an increased interaction between NADPH-cytochrome P450 oxidoreductase and cytochrome P450c17. These studies linked cytochrome b_5 to the cytochrome P450 monooxygenase system.

6.7.2 Mechanism of action of cytochrome b_5

The proposed mechanism of the positive modulating action cytochrome b_5 imposes on the cytochrome P450 monooxygenase system is summarised below.

6.7.2.1. Transfer of second electron

After studying the addition of NADH to a microsomal system, Hildebrandt and Estabrook (23) concluded that the second and rate-limiting electron in the cytochrome P450 reaction cycle could be provided by cytochrome b_5 . In microsomal assay systems NADH has a synergistic effect on the hydroxylation of many cytochrome P450 substrates. In reconstituted systems, this was shown to be mediated by the cytochrome b_5 -cytochrome b_5 reductase pathway (46). With cytochrome P450 reductase being able to reduce cytochrome b_5 , neither cytochrome b_5 reductase nor NADH were necessary in most cases for the stimulation of the reaction. Electrons could thus flow from NADPH (or NADH), through cytochrome P450 reductase, to cytochrome b_5 and cytochrome P450 (47) (figure 6.8). Thermodynamically, it is highly unlikely that cytochrome b_5 will be able to reduce the ferric form of cytochrome P450. However, the oxyferrous form (one electron reduced form), with its midpoint potential of ~50 mV, can readily accept the second electron in the catalytic cycle from cytochrome b_5 (48).

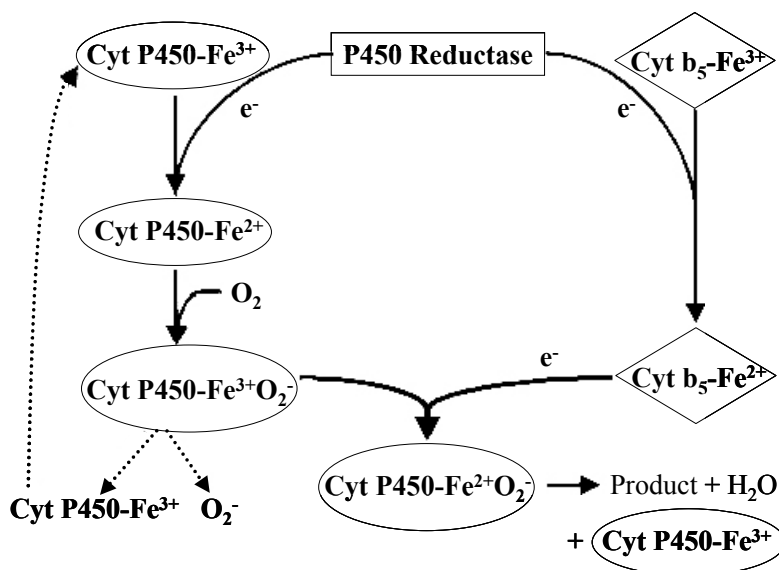


Figure 6.8 Schematic representation of the input of the second electron of cytochrome P450, facilitated by cytochrome b₅. The dotted line indicates the decreased uncoupling pathway during enhanced coupling (49).

6.7.2.2. Enhancement of coupling

In addition to the products of monooxygenation, hydrogen peroxide is produced during the catalysis of the monooxygenation of various substrates (50). The addition of cytochrome b₅ causes enhanced coupling of NADPH oxidation to substrate oxidation resulting in a decrease in the release of superoxide, which dismutates to hydrogen peroxide. At the same time it does not cause an increase in the consumption of NADPH, but rather a decrease, thereby increasing the formation of product (51). Stoichiometric measurements have related NADPH and oxygen consumption with hydrogen peroxide and product formation and shown that the addition of cytochrome b₅ enhances product formation at the expense of hydrogen peroxide formation (52) (figure 6.8). It is thought that cytochrome b₅ provides the second electron faster than the rate of superoxide anion release (uncoupling) and thus allowing more product to be formed.

6.7.2.3. Two electron acceptor complex

Cytochrome b₅ forms a tight, equimolar complex with cytochrome P450 through ionic interactions between the heme and polypeptide carboxyl groups of cytochrome b₅ and positively charged residues of cytochrome P450 (52). This complex can accept two electrons during a single interaction with NADPH cytochrome P450 reductase. As soon as cytochrome P450 is reduced by the reductase, cytochrome b₅ rapidly takes the electron from the ferrous cytochrome P450 enabling cytochrome P450 to accept a second electron immediately. Before

dissociating from the complex, cytochrome P450 reductase can therefore transfer two electrons rapidly. The oxycytochrome P450 oxidises the ferrous cytochrome b_5 when cytochrome P450 interacts with oxygen (figure 6.9). In the absence of cytochrome b_5 , cytochrome P450 would have to dissociate from the reductase, interact with the oxygen and subsequently interact for a second time with NADPH cytochrome P450 reductase (49, 53).

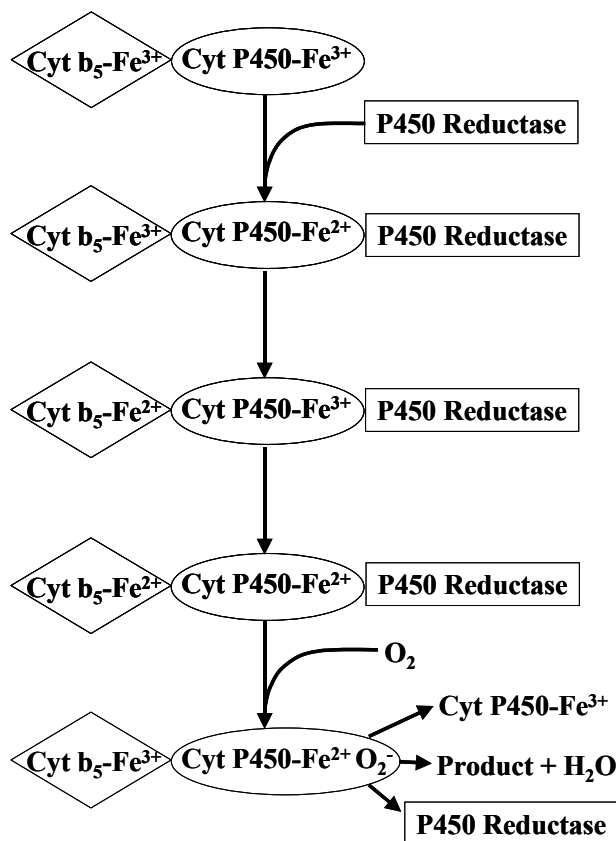


Figure 6.9 Suggested two-electron acceptor complex consisting of cytochrome b_5 bound to cytochrome P450 (49).

6.7.2.4. Cytochrome b_5 -dependent allosteric influence

The suggested role of cytochrome b_5 as effector in the cytochrome P450 monooxygenase reactions is based partly upon the facilitation by cytochrome b_5 in the breakdown of the oxycytochrome P450 complex into its products and ferricytochrome P450. Cytochrome b_5 (and putidaredoxin) was shown to be such an effector for oxycytochrome P450_{cam}, irrespective whether the effector was in the oxidised or reduced state (54). In the absence of an effector, oxycytochrome P450_{cam} auto-oxidized to ferric cytochrome P450 and superoxide. Apo-cytochrome b_5 was shown to be just as effective as an effector as the holo-cytochrome b_5 (54).

Another system where this effect was shown, was in cytochrome P450_{3A4} catalysed reactions (55). Here too apo-cytochrome b_5 was just as effective in supporting the reduction of cytochrome P450_{3A4} and product formation as holo-cytochrome b_5 (56). This indicated that cytochrome b_5 did not need to undergo redox changes or to transfer an electron to cytochrome P450.

The stimulation of 17,20-lyase activity of human cytochrome P450_{C17} by cytochrome b_5 follows a similar trend. Miller *et al.* (57) hypothesised that human cytochrome b_5 acts principally as an allosteric effector which interacts primarily with the cytochrome P450_{C17} oxidoreductase complex to stimulate 17,20-lyase activity without direct electron transfer. NADPH donates two electrons to the FAD domain of cytochrome P450 oxidoreductase (in contact with the microsomal membrane), which passes the electrons to the FMN moiety. The FMN domain of oxidoreductase, which is connected to the FAD domain by a connecting domain and a hinge region, must rotate $\sim 90^\circ$ to dock with the redox-partner binding site of cytochrome P450_{C17}. The interaction of cytochrome P450_{C17} and oxidoreductase is adequate to support 17 α -hydroxylation, but the complex rarely adopts the geometry required to catalyse the 17,20-lyase reaction. The presence of either holo- or apo-cytochrome b_5 favours the interaction of oxidoreductase and cytochrome P450_{C17} in an orientation that satisfies the more stringent conformation restrictions required by the 17,20-lyase reaction, facilitating productive electron transfer from oxidoreductase to cytochrome P450_{C17} and subsequent catalysis. The precise site(s) of action of cytochrome b_5 remains unknown (57) (figure 6.10).

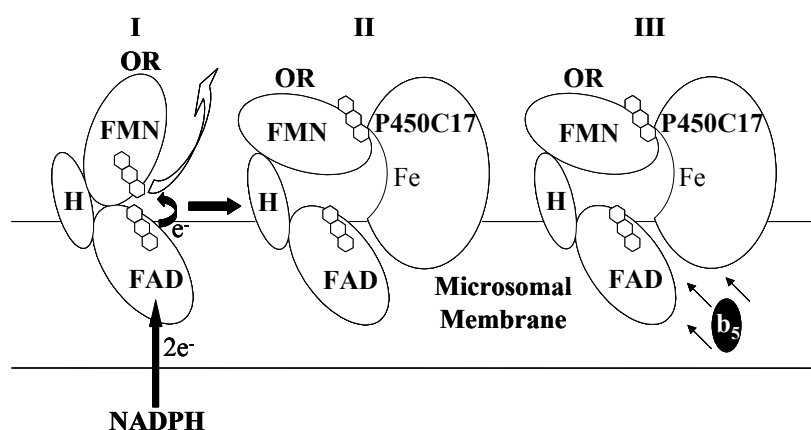


Figure 6.10 Proposed function of cytochrome b_5 in cytochrome P450_{C17} activity. I: The oxidoreductase complex receives two electrons from NADPH. II: The FMN moiety rotates to be able to dock with its redox partner to facilitate 17-hydroxylation activity. III: The presence of cytochrome b_5 enables the oxidoreductase and C17 to adopt the more stringent conformation required for 17,20-lyase activity (57).

A report in 2001 challenged the allosteric effect of apo-cytochrome b_5 on cytochrome P450 $3a_4$ (58). The stimulatory effect observed for apo-cytochrome b_5 was attributed to the excess heme, present in the cytochrome P450 $3a_4$ (and cytochrome P450 c_{17}) preparation, converting the apo- form to the stimulatory holo-form of cytochrome b_5 . Excess heme in the cytochrome P450 $3a_4$ preparation amounted to ~35% of the cytochrome P450 level. The stimulatory effect of apo-cytochrome b_5 was removed when a heme scavenger, in the form of apo-myoglobin, was added (58). However, another report, re-examining the stimulatory effect of apo-cytochrome b_5 on cytochrome P450 $3a_4$, showed an excess heme of only ~5%, which cannot account for the observed decrease in the stimulatory effect. Furthermore it was shown that the addition of apo-myoglobin did not inhibit the stimulatory effect (59). Although the detailed mechanism remains unknown, it seems as if the apo-cytochrome b_5 does have an effect on cytochrome P450 activity.

1.8 INFLUENCE OF CYTOCHROME b_5 ON STEROIDOGENESIS

6.8.1 Human steroidogenesis

In steroidogenesis, cytochrome P450 c_{17} , can either hydroxylate its substrate to form precursors for glucocorticoids, or form precursors for sex steroids through its lyase activity. Cytochrome b_5 has been suggested to contribute in the differential regulation of these two pathways by promoting the lyase activity in reproductive tissues. Cytochrome b_5 is expressed in high levels in tissues such as the testes and the *zona reticularis* of the adrenals (34).

Aldosterone, cortisol and the so-called adrenal androgens, dehydroepiandrosterone (DHEA) and DHEA sulphate (DHEAS) are synthesised in the human adrenal cortex. In the adult adrenal gland, the *zona glomerulosa* produces aldosterone, the *zona fasciculata* cortisol and the *zona reticularis* both DHEA and DHEAS (60). The enhancement of 17,20-lyase activity by cytochrome b_5 to form DHEA or androstenedione is well documented. Interestingly cytochrome b_5 also stimulates the formation of androstenedione from 17 α -hydroxyprogesterone. In humans the substrate for the 17,20-lyase reaction is almost exclusively 17 α -hydroxypregnenolone with negligible androstenedione formation from 17 α -hydroxyprogesterone. This enhancement of 17 α -hydroxyprogesterone metabolism and subsequent androstenedione formation by cytochrome b_5 may possibly prevent the build-up of a dead-end product which may even inhibit cytochrome P450 c_{17} (61,62).

The fetal human adrenal is histologically different from the adult adrenal with respect to the zonation of the gland. It can be divided into the fetal zone, the neocortex (also called the definitive zone) and the transitional zone. The fetal zone is found only during the fetal development and is responsible for the high steroidogenic capacity of the fetal adrenal. The neocortex develops into the cortical tissue of the adult adrenal. The transitional zone is hypothesised to produce cortisol during the last part of gestation. Cytochrome b_5 synthesis was observed in the dehydroepiandrosterone sulphate (DHEAS) producing fetal zone throughout gestation as well as in the transitional zone. Significant cytochrome b_5 synthesis could not be detected in the neocortex until the second half of gestation (63).

Postnatally, the neocortex and transitional zone expand to form the *zona glomerulosa* and the *zona fasciculata*. The fetal zone involutes, which coincides with a drop in circulating levels of DHEA(S) for the period of one to five years of age. The *zona reticularis* of the human adrenal at this age seems to be poorly developed. From the age of five to seven years, the *zona reticularis* starts to expand and continues through puberty, with an increase in cytochrome b_5 synthesis. This high level of cytochrome b_5 synthesis is maintained through adulthood (60). It can thus be concluded that the relative synthesis of cytochrome b_5 appears to be paramount in determining the adrenal capacity in DHEAS production.

Other adult steroidogenic tissues where cytochrome b_5 was co-localized with cytochrome P450c17, were the Leydig cells of the testes, theca internal cells of the follicle, theca lutein cells and isolated cell clusters in the ovarian stroma, although co-localization of cytochrome b_5 and cytochrome P450c17 were shown in the Leydig cells of the fetal testes, it could not be detected in the midgestational fetal ovary (64). The presence of cytochrome b_5 and cytochrome P450c17 in isolated cell clusters in the ovarian stroma may account for continued post-menopausal androgen production in the ovary. Midgestational ovaries only produce nominal amounts of steroids (65), which correlates with the observed low immunoreactivity for cytochrome b_5 and cytochrome P450c17. The presence of both enzymes in the Leydig cells of the human fetal testes may provide a possible explanation for the observed increase in catalytic efficiency for the $\Delta 5$ -steroidogenic pathway in this tissue (66).

6.8.2. Guinea pig steroidogenesis

Cytochrome b_5 may control the dual activities of cytochrome P450c17 in guinea pig adrenal steroidogenesis since the lyase activity decreased when the cytochrome b_5 was removed from the cytochrome P450 fraction in guinea pig adrenal microsomes (67). Shinzawa *et al.* (68)

suggested that the effect of added cytochrome b_5 on the cytochrome P450c17 catalytic activity was not due to electron transfer, but rather due to a shift in optimal pH for the two reactions being catalysed. They observed a shift in the optimal pH for the 17α -hydroxylation reaction, pH ~ 7 , and for the lyase reaction, pH ~ 6.6 . In contrast, they showed that both activities decreased with the addition of cytochrome b_5 at a pH < 5.3 .

6.8.3. Porcine steroidogenesis

16-Androstene steroids are C19 steroids, produced primarily by the Leydig cells in porcine testis. They enter circulation via the spermatic vein and are subsequently concentrated in the fatty tissue (69). Androstenedione, a C19 steroid, has a distinctive strong musk-like odour and is the primary cause for 'boar taint' (70). Currently, most pig boars in North America are castrated to avoid boar taint, which leads to complications, as these animals grow at a slower rate.

The first step in the synthesis of 16-androstene steroids from pregnenolone is the formation of 5,16-androstadien-3 β -ol. This reaction is catalysed by the andien- β synthase system in a cytochrome P450-dependent reaction. It has been shown that cytochrome b_5 is essential for andien- β synthase activity *in vivo* (69) and that high levels of cytochrome b_5 have a stimulatory effect on adrenal steroidogenesis (71). Two immunoreactive cytochrome b_5 species have been found in the porcine testis (70). One of these has a molecular mass that correlates with soluble cytochrome b_5 ($\sim 12,000$ Da), while the other correlates with intact testicular cytochrome b_5 ($\sim 16,000$ Da). It has been shown that an increase in cytochrome b_5 levels, in particular the low molecular weight species, is associated with a higher level of 16-androstene production in porcine testis (70). This is the first suggestion that the soluble-like form of cytochrome b_5 plays a role in steroidogenesis. No correlation could be drawn between the levels of the lower molecular weight form and the sexual maturity of the pigs. It can thus be concluded that the levels of this form of cytochrome b_5 are determined genetically. In comparison to the adrenal gland, the cytochrome b_5 content in the testis is about five times higher than that in the adrenal gland (33) (Table 6.1). This is also an indication of the role cytochrome b_5 plays in androgen synthesis.

Table 6.1 Levels of cytochrome P450 and cytochrome b_5 in porcine adrenal and porcine testicular microsomes (33). ^a CO difference spectra ; ^b Enzyme assays.

Enzyme	Adrenal	Testis
Cytochrome P-450 (nmol/mg protein) ^a	0.78	0.72
Cytochrome b_5 (nmol/mg protein) ^b	0.16	0.84

6.8.4. Rat steroidogenesis

As is evident, cytochrome P450c17 catalyses the reactions at the branch point of glucocorticoid and sex hormone biosynthesis. Glucocorticoids play an important role in stress responses, carbohydrate metabolism and general metabolite homeostasis. Androgens on the other hand play an important role in reproductive biology, such as the development of secondary male characteristics. Cytochrome P450c17 is expressed in the adrenal of most species, but is absent in rat, mouse and rabbit adrenals (72). Treatment with ACTH though stimulates the rabbit adrenal to produce cytochrome P450c17 (73). In rat adrenals corticosterone is produced as the main glucocorticoid in the absence of cytochrome P450c17. No androgens or cortisol is synthesised.

In rat testis the outer mitochondrial membrane form of cytochrome b_5 was detected in both the mitochondria and endoplasmic reticulum. No microsomal cytochrome b_5 could be detected in rat testes. Mitochondrial outer membrane cytochrome b_5 was co-localized with cytochrome P450c17 in the Leydig cells of the rat testes. The influence of the microsomal form on the catalytic activity of cytochrome P450c17 was different from that observed with the mitochondrial outer membrane form of cytochrome b_5 (74). Mitochondrial outer membrane cytochrome b_5 exhibited a preferential elevation of the 17α -hydroxylation activity in a concentration-dependent manner. The microsomal form showed a preference for the lyase activity at low concentrations. At progesterone concentrations comparable to the physiological cholesterol availability, mitochondrial outer membrane cytochrome b_5 elevated the production of 17α -hydroxyprogesterone primarily and subsequently facilitated the conversion of the formed intermediate to androstenedione. It thus seems that the mitochondrial outer membrane form, rather than microsomal cytochrome b_5 , serves as an activator for androgen biosynthesis in rat Leydig cells (74).

6.8.5. Sheep adrenal steroidogenesis

In a study by Swart *et al.* (75) it was shown that the addition of cytochrome b_5 had an inhibitory effect on cytochrome P450c17 while the activity of cytochrome P450c21 appeared

to be unchanged. These results were in contrast to results obtained with guinea pig microsomes where both cytochrome P450c17 and cytochrome P450c21 activities were stimulated by the addition of cytochrome b₅. In a subsequent study it was shown that the presence of cytochrome b₅ in ovine adrenal microsomes did not confer lyase activity on the ovine adrenal cytochrome P450c17 (76). As mentioned previously, earlier studies have shown that cytochrome b₅ has the ability to amplify greatly the lyase activity of cytochrome P450c17 not only in humans (77, 78) but also in other species (79). Cytochrome b₅ is present in ovine adrenal microsomes at a concentration of 0.22 nmol/mg protein and the cytochrome P450:cytochrome b₅ ratio is approximately 5:1, this ratio compares well to figures previously reported for ovine gonadal tissue where adequate Δ^5 -lyase activity was demonstrated (80). Immunostaining analysis of ovine adrenal microsomes showed that cytochrome b₅ is present in comparable amounts in ovine testes, adrenal and liver tissue (Chapter 4, figure 4.3). The maintenance of a tetrameric state after SDS PAGE employing standard denaturing conditions, suggest strong quaternary structural interactions and resistance to anionic detergents and mercaptoethanol. In addition the signal obtained for cytochrome b₅ in ovine tissue exhibits the same intensity of the tetrameric aggregate as bovine adrenal microsomal cytochrome b₅ shown in the same blot.

It is evident that ovine adrenal microsomes could not convert pregnenolone or progesterone to C₁₉-androgen precursors (76). However, the absence of cytochrome P450c17 lyase activity towards Δ^5 -steroids does not occur due to a lack of cytochrome b₅ in the ovine adrenal cortex. It can therefore be deduced that the inability of sheep cytochrome P450c17 to exhibit lyase activity is due to the presence or absence of a modulating substance other than cytochrome b₅ or that the concentration of a certain aggregation state of cytochrome b₅ (monomeric, dimeric or tetrameric) might be critical for lyase activation. As a significant portion of the work described in this section was carried out as part of this PhD study, the paper containing the full text of these findings is included in Appendix B. In this paper (76) the purification, characterisation and quantification of cytochrome b₅ in sheep adrenals as well as the immunological study was a part of this PhD investigation.

The results obtained from our previous investigation into the hydroxylase and lyase activity of ovine cytochrome P450c17 prompted a further investigation into the influence of purified and trypsin truncated cytochrome b₅ on sheep adrenal steroidogenesis.

1.9 AN INVESTIGATION INTO THE INFLUENCE OF PURIFIED CYTOCHROME b_5 ON OVINE ADRENAL STEROIDOGENESIS

6.9.1. Introduction

The aim of this study was to investigate the influence of purified cytochrome b_5 on sheep adrenal steroidogenesis. In an earlier study the question was raised whether cytochrome b_5 exists in a monomeric or multimeric form *in vivo* (81). The intact and trypsin truncated forms of cytochrome b_5 were subsequently prepared for use in an adrenal microsomal assay system. The truncated cytochrome b_5 remains active since the proteins contains the heme moiety as previously shown with absorption spectra and ESMS analyses. The possibility of direct electron transfer by cytochrome b_5 in adrenal steroidogenesis can therefore be investigated using the truncated form of cytochrome b_5 .

6.9.2. Experimental

6.9.2.1 Preparation of ovine adrenal microsomes. Ovine adrenal microsomes were prepared as previously described (82). Cytochromes P450 and b_5 concentrations of the adrenal microsomes were determined according to the methods described by Estabrook and Werringloer (83). Full length as well as trypsin truncated ovine cytochrome b_5 were prepared as described in Chapter 3.

6.9.2.2 Steroid metabolism assay. Pregnenolone and progesterone conversion was assayed in adrenal microsomes as described by Swart *et al.* (75).

The appropriate volume of progesterone or pregnenolone were pipetted onto small filter paper squares to yield a final concentration of 6 μ M. Tritiated progesterone or pregnenolone in ethanol was pipetted onto the same filter paper to yield a total count of 100,000 cpm/ml in the final reaction mixture. The filter paper squares were dried under a stream of nitrogen gas in 5 ml disposable glass tubes.

All reactions were carried out in 50 mM Tris buffer, pH 7.4, containing 1% BSA and 50 mM NaCl. Reaction mixtures contained $MgCl_2$ (10 mM), isocitrate (2 mg/ml), NADPH (1 mM), isocitrate dehydrogenase (0.2 U/ml) and microsomal suspension (0.35 μ M cytochrome P450). All the components were pipetted onto the dried filter paper, with the exception of the isocitrate dehydrogenase and the NADPH, and pre-incubated for 5 minutes at 37°C in a water bath with constant agitation. The reaction was initiated by the addition of the isocitrate dehydrogenase and NADPH to yield a final reaction volume of 0.5 ml. Aliquots of 50 μ l of

the reaction mixture were removed at specific time intervals and added to 450 µl distilled water and 5 ml cold methylene chloride. The mixture was vortexed and immediately placed on ice. Steroids were vortexed for 1 minute in the methylene chloride/water mixture followed by centrifugation to separate the organic and aqueous phases. The aqueous phase was aspirated off and the organic phase dried under a stream of nitrogen. The residue was redissolved with 100 µl HPLC grade methanol. These samples were analysed on a Novapak® HPLC C-18 column on a Waters (Milford, MA) high performance liquid chromatograph coupled to a WispTM automatic injector (Waters) and a Flo-one liquid scintillation spectrophotometer (Radiomatic, FL).

The mobile phase for the HPLC analyses consisted of solvent A (35% H₂O:65% methanol) and solvent B (100% methanol). The solvent gradient formation for the HPLC analyses is depicted in table 6.2.

Table 6.2: Chromatographic gradient elution of steroids.

Time (min)	Flow (ml/min)	% A	% B	Curve Type
0.00	1.00	100	0	6
8.00*	1.00	100	0	6
15*	1.00	100	0	6
20*	1.00	0	100	6
25*	1.00	0	100	6
30.00	1.00	100	0	6
35.00	1.00	100	0	6
* Radioactive flo detector and scintillation fluid pump on				

6.9.2.3 Difference spectra. Difference spectra were recorded according to the method described by Estabrook and Werringloer (83).

6.9.3. Results

The cytochrome P450 and cytochrome b_5 concentrations in the adrenal microsomes were spectrophotometrically assayed. Cytochrome P450 in CO-sparged microsomal samples were reduced with sodium dithionite and cytochrome b_5 with NADH. Difference spectra were recorded (figure 6.11) from which the concentrations were determined by applying the respective milimolar extinction coefficients.

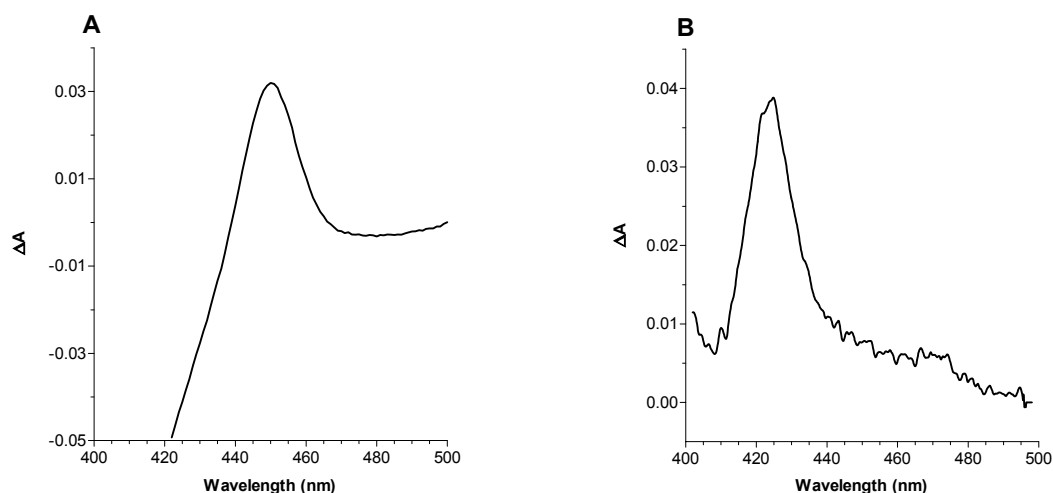


Figure 6.11 Difference spectra of cytochrome P450 (A), exhibiting an absorption maximum at 450 nm, and cytochrome b_5 (B), exhibiting an absorption maximum at 424 nm, in sheep adrenal microsomes.

HPLC analyses of pregnenolone and progesterone conversion assays are shown in figure 6.12. In the microsomal system pregnenolone was metabolised by cytochrome P450c17, 3β HSD and cytochrome P450c21 to the respective steroid metabolites. Cytochrome P450c17 and cytochrome P450c21 metabolised progesterone to 17OH progesterone, deoxycorticosterone and deoxycortisol respectively. No androstenedione was detected when progesterone was used as substrate.

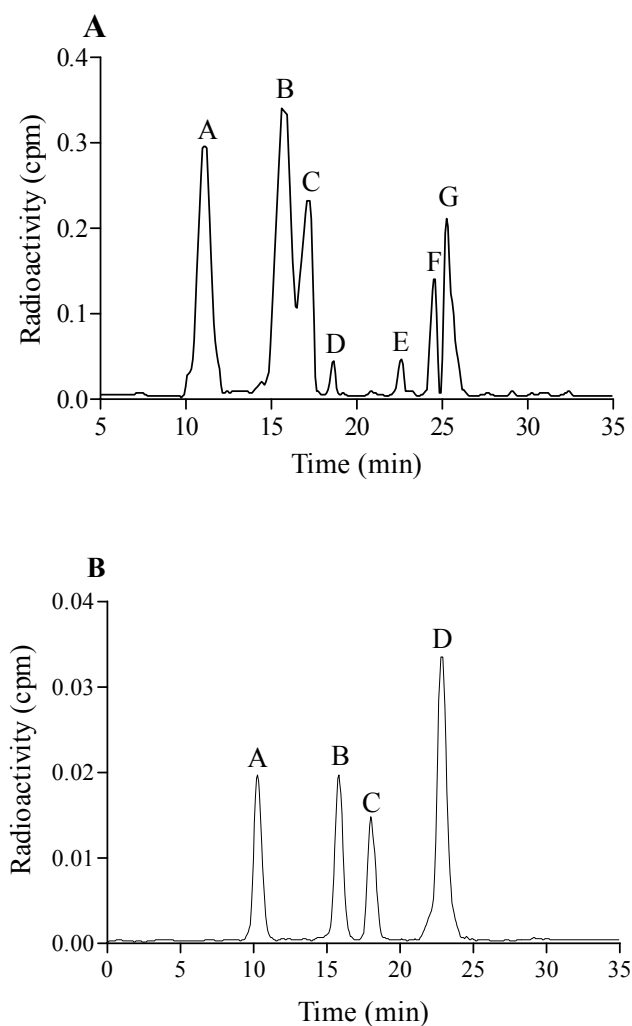


Figure 6.12 **A:** Radio chromatogram of the elution profile of steroid metabolites formed in pregnenolone metabolism (10 μ M). **B:** Radio chromatogram of the elution profile of tritiated steroid metabolites formed in progesterone metabolism (10 μ M). A: Deoxycortisol B: Androstenedione C: Deoxycorticosterone D: 17OH Progesterone E: DHEA & 17OH Pregnenolone F: Progesterone G: Pregnenolone.

Prior to determining the effect of cytochrome b_5 on adrenal steroidogenesis, a pregnenolone conversion assay was carried out. In this assay different cytochrome b_5 concentrations were added to the reaction mixture, thus establishing the cytochrome b_5 concentration affecting the metabolic outcome. Increasing ratios of intact full length cytochrome b_5 :endogenous cytochrome b_5 showed that cytochrome b_5 added to a final concentration of ~ 1 μ M yielded maximum stimulation of steroid conversion (figure 6.13). As the concentration of the added cytochrome b_5 is increased, there is an increase in the levels of androstenedione and a decrease in the levels of deoxycortisol. These results suggest a change in cytochrome P450c17 activity.

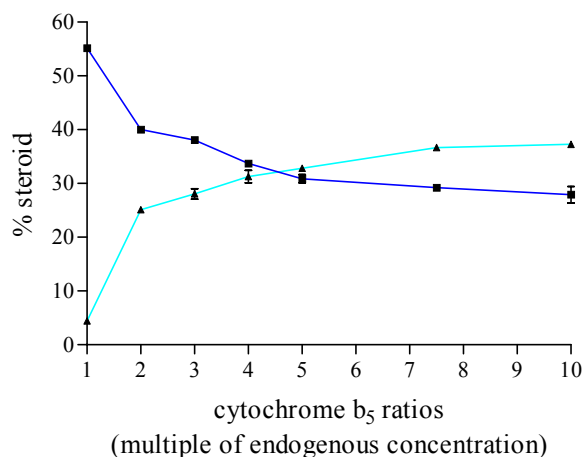


Figure 6.13 Influence of cytochrome b₅ on pregnenolone (10 μ M) conversion. Increasing ratios of intact full length cytochrome b₅, 1:1 to 10:1, was added to the microsomal system. ([P450] = 0.35 μ M; [cytochrome b₅] = 0.07 μ M). ▲ : Androstenedione, ■ : Deoxycortisol. (Error bars indicate \pm SD, n=3)

The influence of cytochrome b₅ on progesterone and pregnenolone metabolism by ovine adrenal microsomes was subsequently investigated. Pregnenolone metabolism supplies all the substrates for the progesterone metabolism pathway. Progesterone metabolism was therefore first characterised before looking at the 17,20-lyase activity of cytochrome P450c17. In adrenal microsomes cytochrome P450c17 converts progesterone to 17 hydroxyprogesterone. Cytochrome P450c21 converts progesterone and 17 hydroxyprogesterone to deoxycorticosterone and deoxycortisol respectively. The metabolism of progesterone, without added intact full length cytochrome b₅ clearly showed that the major metabolite was deoxycortisol (figure 6.14A). Progesterone metabolism was subsequently assayed in the presence of trypsin truncated cytochrome b₅. Although the trypsin truncated cytochrome b₅ contains no membrane binding domain, it is still active with respect to electron transfer. No notable change in progesterone metabolism in ovine adrenal microsomes was observed in the presence of the truncated protein (figure 6.14B).

Addition of intact full length cytochrome b₅ increased the production of deoxycorticosterone ~100%. In the presence of intact full length cytochrome b₅ the ratio of deoxycortisol:deoxycorticosterone was 2, while in the presence of truncated cytochrome b₅ these metabolites were present in an equimolar ratio after 14 minutes (figure 6.14C). The level of 17-OH progesterone was ~60% lower than in the control and in the presence of trypsin truncated cytochrome b₅.

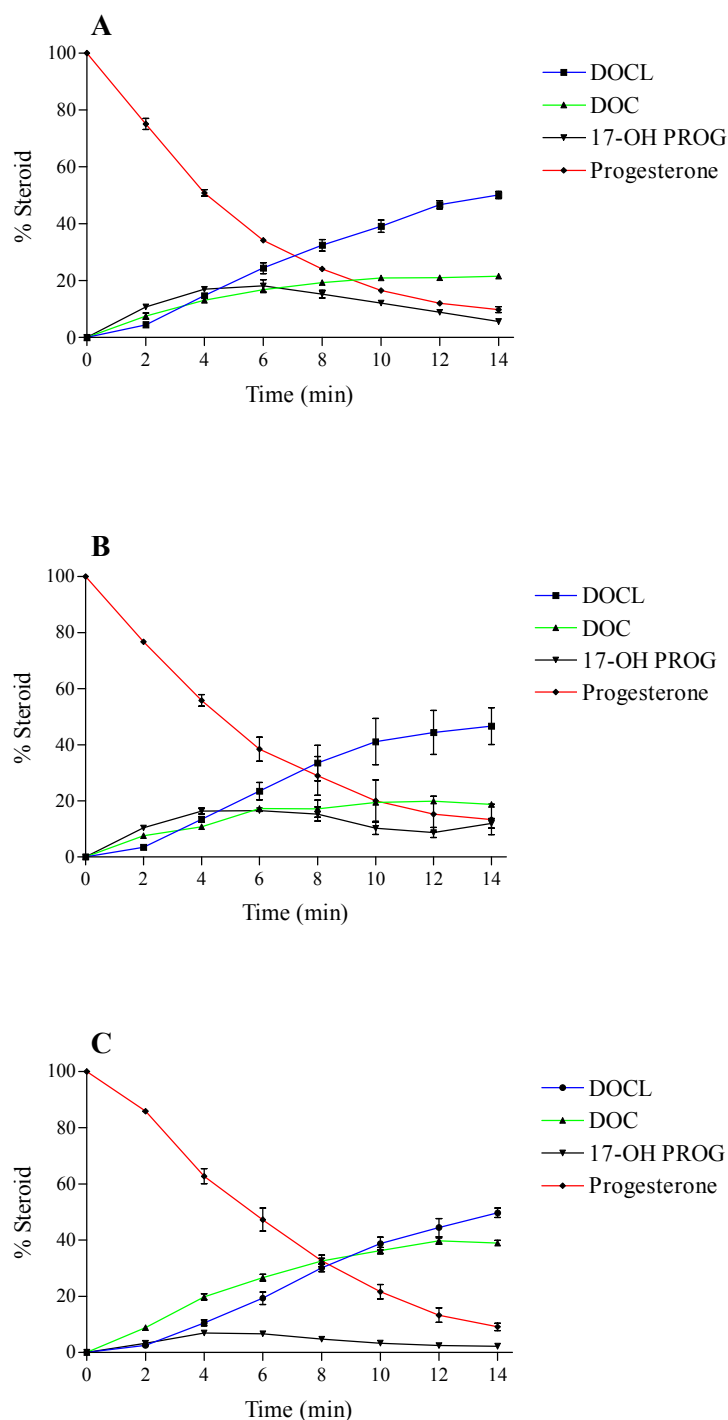


Figure 6.14 Influence of cytochrome b_5 on progesterone ($10\mu\text{M}$) metabolism in adrenal microsomes. A: in the presence of endogenous cytochrome b_5 , B: in the presence of $1\mu\text{M}$ truncated cytochrome b_5 , C: in the presence of $1\mu\text{M}$ intact cytochrome b_5 . DOCL: Deoxycortisol; DOC: Deoxycorticosterone; 17-OH PROG: 17OH Progesterone (Error bars indicate $\pm\text{SD}$, $n=3$)

Pregnenolone metabolism was investigated in the presence of truncated and full length cytochrome b_5 . The addition of truncated cytochrome b_5 had no influence on pregnenolone conversion with the ratio of steroid metabolites remaining the same as in the control reaction (table 6.3).

Table 6.3 Steroids formed after 60 minutes in pregnenolone conversion assay.

	Control	Truncated cytochrome b_5 added	Intact cytochrome b_5 added
Deoxycortisol	58%	57%	27%
androstenedione	7.0%	7.1%	37%
Deoxycorticosterone	6.3%	6.3%	3.9%
17 Hydroxy progesterone	1.4%	1.5%	4.9%
17 Hydroxy pregnenolone and dehydroepiandrosterone	3.3%	3.1%	5.3%
Progesterone	12%	12%	6.3%
Pregnenolone	12%	12%	16%

In the absence of added cytochrome b_5 , it can be seen that deoxycortisol is the major metabolite at the end of the incubation period (figure 6.15A). It was not possible to separate dehydroepiandrosterone (DHEA) and 17-OH pregnenolone on the chromatographic system used to separate the steroid metabolites. Both components eluted as a single peak and are thus indicated together in the results. It is worthy to note that at five minutes with 80% of the pregnenolone substrate metabolised the DHEA/17-OH pregnenolone fraction constitutes the largest portion of the steroid metabolites and decreases thereafter. Negligible amounts of androstenedione are, however, formed.

Deoxycortisol is the major steroid metabolite formed when pregnenolone is metabolised in the presence of endogenous cytochrome b_5 . Upon the addition of full length cytochrome b_5 , deoxycortisol levels decrease and an increase of ~30% is observed in the androstenedione formation (figure 6.15B).

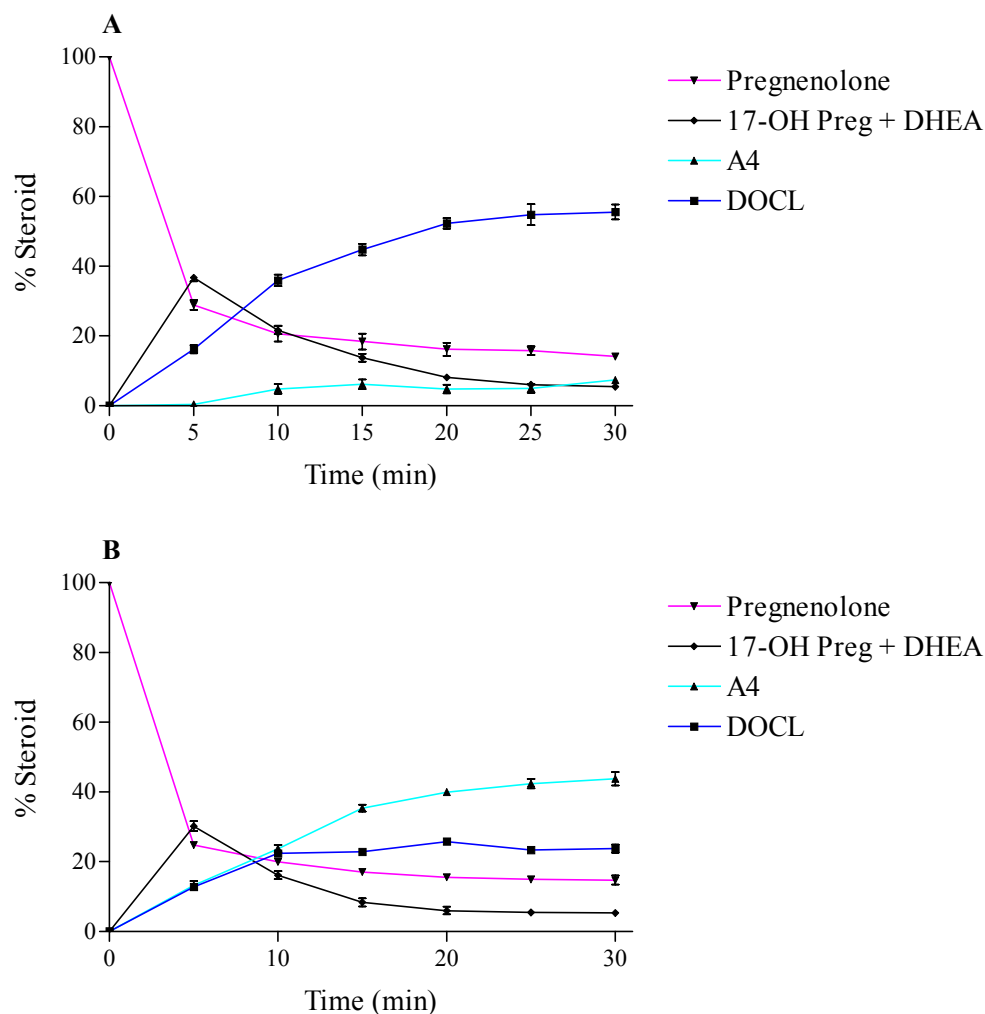


Figure 6.15 Influence of cytochrome b_5 on pregnenolone (10 μ M) metabolism in adrenal microsomes. A: no added cytochrome b_5 and B: addition of 1 μ M intact full length cytochrome b_5 . 17-OH Preg: 17 hydroxy pregnenolone; DHEA: dehydroepiandrosterone; A4: androstenedione and DOCL: deoxycortisol (Error bars indicate \pm SD, $n=3$)

6.9.4. Discussion

Earlier work by Meadus *et al.* (69) on porcine and bovine steroidogenesis showed that the cytochrome P450 to cytochrome b_5 ratio in testicular tissue was 1:1 and 5:1 respectively in the adrenal gland. This ratio is in line with the value obtained in the ovine adrenal microsomes used in this study. The ratio of cytochrome P450 to cytochrome b_5 in the ovine adrenal microsomes used were 5:1 (14.95 μ M:3.19 μ M). In this study the addition of cytochrome b_5 to a 1:2 (cytochrome P450:cytochrome b_5) ratio yielded maximum stimulation of steroid conversion and subsequently influenced the adrenal steroid metabolite profile. At higher cytochrome b_5 levels ($>1:10$) an inhibitory effect was observed (results not shown). A similar inhibitory effect was observed in both human and yeast microsomes, although at

ratios of cytochrome P450: cytochrome b₅ higher than 1:30 (57). This inhibitory effect was
 s and cytochrome P450.

It is apparent from results shown in figure 6.13 that cytochrome b₅ has a profound effect on adrenal steroidogenesis. Analyses of pregnenolone metabolites showed that there was a decrease in deoxycortisol production with a concomitant increase in androstenedione production as the cytochrome b₅ levels were increased. At a cytochrome b₅:endogenous cytochrome b₅ ratio of 7:1 to 10:1 maximum stimulation of the lyase activity was observed.

During the metabolism of Δ^4 -steroids, the ratio of deoxycorticosterone increased with the addition of cytochrome b₅. While the 17 hydroxyprogesterone metabolite decreased, the amount of deoxycortisol formed remained unchanged, suggesting that the cytochrome P450C21 activity is stimulated by cytochrome b₅ and cytochrome P450c17 hydroxylase activity is unaffected. These results were similar to those obtained earlier by Swart *et al.* (75). During the metabolism of Δ^4 -steroids no androstenedione was detected, confirming the absence of 17,20-lyase activity of ovine cytochrome P450c17 in Δ^4 -steroid conversions. This may be due to the fact that the 17,20-lyase catalytic efficiency with a Δ^4 -substrate is nearly a hundred fold slower than the same reaction with a Δ^5 -substrate (57). The trypsin truncated form of cytochrome b₅ did not have any effect on ovine adrenal microsomal steroidogenesis. This result reiterates the importance of the membrane binding domain of cytochrome b₅ for normal protein-protein interaction as far as this hemoprotein is concerned. Recombinant DNA-technology is often an attractive, and sometimes essential, alternative to the isolation and purification of proteins from animal or plant tissue. The cloning strategies used often dictate modifications specifically of the membrane binding domains of proteins, facilitating heterologous expression and/or subsequent purification. This study has shown that the deletion or modification of the membrane binding domain of cytochrome b₅ has a marked influence on the interaction of the modified protein with itself and other proteins, thus affecting the outcome of steroidogenesis. It is therefore important that this aspect should be considered when making deductions pertaining to structure-function relationship of cytochrome b₅ when using recombinant modified cytochrome b₅ and its associated antibodies.

It is evident from the data presented that the addition of full length cytochrome b₅ to ovine adrenal microsomes stimulates the 17,20-lyase activity of cytochrome P450c17 in Δ^5 -steroid metabolism. Conversion of pregnenolone to progesterone is negligible both in the control and

in the presence of added cytochrome b_5 . The 17-OH pregnenolone intermediate formed in the absence of added cytochrome b_5 is converted to 17-OH progesterone and subsequently to deoxycortisol, the major metabolite formed. The decrease in the 17OH-pregnenolone/DHEA fraction and the low levels of androstenedione formed shows that the lyase activity in the absence of added cytochrome b_5 was low, favouring the 3β HSD and cytochrome P450c21 catalyses of 17-OH pregnenolone. Addition of 1 μ M of cytochrome b_5 yielded a dramatic increase in androstenedione production with a concomitant decrease in deoxycortisol production. The decrease 17OH-pregnenolone/DHEA fraction together with the decrease in the formation of deoxycortisol is most probably due to the stimulation of the lyase activity of cytochrome P450c17.

It is clear, from the data presented in this study, that cytochrome b_5 has an effect on ovine adrenal steroidogenesis at higher than physiological cytochrome P450:cytochrome b_5 ratios. These results are similar to results obtained in other species (84).

Although we showed the stimulatory effect of cytochrome b_5 on the lyase activity of cytochrome P450c17, it remains uncertain whether the monomeric and aggregated forms of cytochrome b_5 both have the same effect on the lyase activity. Engelbrecht (85) showed, in Western blot analyses, that antibodies recognising only the aggregate form of cytochrome b_5 also recognised cytochrome b_5 in ovine adrenal tissue sections. Future studies selectively binding cytochrome b_5 using monomer- and aggregate-specific antibodies in adrenal metabolic assays, would lead to a better understanding of the conformation of the active protein *in vivo* and its subsequent influence on adrenal steroidogenesis.

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CHAPTER 7

DISCUSSION

In 1954 Chance and Williams (1) reported the presence of a hemoprotein in the postmitochondrial fraction of rat liver homogenate. This hemoprotein, previously known as cytochrome m, was subsequently renamed cytochrome b₅. Several years later, in 1963, Cooper *et al.* (2) showed that cytochrome b₅ was also present in adrenalcortical microsomes and that it was reduced by NADH and NADPH. Although cytochrome b₅ is found in the cytoplasm of all cells, the highest concentration is found in liver cells. Besides animal tissue, cytochrome b₅ can also be found in plants and fungi (3).

Cytochrome b₅ is an ubiquitous electron transport protein found as both a membrane bound and soluble form. Two membrane bound forms are found, viz. a mitochondrial outer membrane associated form and an endoplasmic reticulum, or microsomal form. Although the two forms are almost identical, they target different organelles. It was shown that a cytochrome b₅ molecule with a net negative charge at its carboxyl-terminal targets the protein to the endoplasmic reticulum while a net positive charge targets the protein to the mitochondria (4). The membrane bound form consists of a hydrophilic catalytic domain and a hydrophobic membrane anchoring domain, connected by a hinge region. Membrane bound cytochrome b₅ plays an important role in various biochemical reactions, such as fatty acid biosynthesis, hepatic cholesterologenesis, N-glycolylneuraminic acid biosynthesis and steroidogenesis. A high degree of homology exists in the structures of soluble cytochrome b₅ and the catalytic domain of the membrane bound form (5). Soluble cytochrome b₅ contains no membrane binding domain and is mainly found in the erythrocytes where it functions in maintaining haemoglobin in a reduced state.

Consisting of six α -helices and five β -strands, cytochrome b₅ is a small cylindrical protein. As mentioned, cytochrome b₅ is folded into two domains. The heme containing hydrophilic catalytic domain, comprising the residues at the amino end of the protein, is the larger of the two domains. A hydrophobic pocket containing the heme, with two well conserved histidine side chains co-ordinating the heme iron, is located in this domain. This heme cleft is flanked by four α -helices with the heme periphery exposed to the environment. On the carboxyl end of the protein, a proline-containing hinge region of ~7 amino acids link the hydrophobic membrane binding domain and the catalytic domain (5). Proteases can digest the protein before or after the hinge region, releasing a soluble catalytic domain. A sequence of ten

amino acids at the carboxyl terminal, is responsible for membrane targeting, discriminating between the mitochondrial outer membrane and the microsomal membrane (6).

The physiological role of cytochrome b_5 in the various biochemical processes is relatively similar in most species studies thus far, with the exception of steroidogenesis, where major differences have been reported in mammals.

The first sheep liver cytochrome b_5 study carried out in our laboratory showed that cytochrome b_5 most probably exists as a tetrameric aggregate *in vivo*, with a relative molecular mass of ~60,000 Da (7). Following up this work, the membrane binding domain was removed by means of tryptic digestion in order to solubilise the protein. The trypsin-solubilised cytochrome b_5 had a relative molecular mass of ~10,180 Da (8). A subsequent study on the purification and characterisation of intact sheep liver cytochrome b_5 reported that the relative molecular mass of cytochrome b_5 is $16,200 \pm 500$ Da, as determined by SDS PAGE (9). These observations prompted our investigation into the characterisation of the native form of ovine adrenal cytochrome b_5 and its influence on adrenal steroidogenesis.

Upon repeating the isolation of sheep liver cytochrome b_5 , as described by Engelbrecht (7), the majority of the isolated cytochrome b_5 was found to be in an aggregated form when analysed on SDS PAGE. High concentrations of mercaptoethanol and prolonged exposure to temperatures greater than 100°C could not dissociate the aggregate completely. An alternative method for the isolation of cytochrome b_5 was subsequently investigated to eliminate the possible formation of aggregates due to the method of isolation.

Liver microsomes were prepared by means of polyethylene glycol precipitation, as alternative to the calcium chloride precipitation method previously used. In addition, the chromatographic conditions used were modified with respect to the anion exchange columns and buffers. The method proved to be more efficient resulting in consistently high yields of cytochrome b_5 . Purified ovine cytochrome b_5 dissolved in 0.25% sodium deoxycholate was predominantly in the monomeric state. This observation correlated with published results where only a monomeric specie was observed in the presence of high levels of detergent (9). In the absence of sodium deoxycholate, the aggregate form was the predominant specie as observed by SDS PAGE analyses. These results satisfied the criteria set out in the second goal of this study.

Although it was thus possible to obtain intact monomeric cytochrome b_5 in solution, the levels of sodium deoxycholate was too high to allow the preparation to be used either for raising of an antibody or in biological assays. These findings raised the question whether cytochrome b_5 occurs in a monomeric or aggregated state *in vivo*. Monomeric and aggregated cytochrome b_5 had been observed in SDS PAGE analyses of microsomal samples, indicating that the denaturing conditions were adequate to liberate cytochrome b_5 from the phospholipid bilayer, but inadequate to dissociate the aggregated form of cytochrome b_5 .

Trypsin treatment of cytochrome b_5 , according to the method of Omura and Takesue (10), yielded unsatisfactory results. This method was best suited for type III trypsin and in our hands, the yield of truncated cytochrome b_5 was very low. We developed a new method for the preparation of truncated cytochrome b_5 . The advantages of this method were: (1) it was independent of the type of trypsin used, (2) a significant increase in the truncated cytochrome b_5 yield was obtained, (3) the preparation time was reduced by 75% and (4) immobilisation of the cytochrome b_5 on the ion exchange column during trypsin treatment protected the protein from overexposure to the trypsin.

Q-ToF ESMS identified two truncated forms of cytochrome b_5 with different molecular masses present in solution. A specie with a molecular mass of 10,182 Da, which correlated with the fragment prepared with the method reported by Omura and Takesue (10), and a smaller fragment with molecular mass of 9,816 Da was observed. An ESMS fragmentation study revealed that both species originated from the same protein. Gibson *et al.* (11) also reported two different sized species when digesting rabbit cytochrome b_5 with trypsin. The difference in size was attributed to deamination of the glutamine residue at position 3 at the amino terminus of the protein which most likely occurred during the isolation and purification of the protein. Results obtained during this study indicated a difference in the amino terminal of the two truncated species of ovine cytochrome b_5 . The smaller of the two trypsin-truncated cytochrome b_5 species is thus, according to the results reported by Gibson *et al.* (11), the product of the native form of the protein. The third goal of this study, the successful solubilisation of cytochrome b_5 by means of trypsin truncation and its subsequent characterisation, was thus accomplished.

HP-GPC studies showed that intact cytochrome b_5 existed as both monomer and an aggregate at low salt concentrations. The two isolated species exhibited single peaks when rechromatographed immediately. A 24 hour incubation of the two purified species, however,

resulted in aggregation of the cytochrome b_5 . Intact cytochrome b_5 , when incubated in PBS, exhibited a single peak with a retention time corresponding to that of the aggregate. Trypsin-truncated cytochrome b_5 showed a single peak when analysed on HP-GPC, irrespective of the ionic strength of the buffers used. For immunisation purposes, protein samples were dissolved in PBS, decreasing the possibility of obtaining a monomer specific antiserum. Since part of the fourth goal of this study was to prepare an antiserum that would recognise the monomeric form of cytochrome b_5 , raising of an anti-trypsin-truncated cytochrome b_5 antibody was investigated.

Antibodies were raised in rabbits against both the intact full-length and trypsin truncated forms of cytochrome b_5 , using the method described by Bellstedt *et al.* (12). Western blot analyses, using a colorimetric detection of the immunoprecipitate, indicated the presence of cytochrome b_5 aggregate only (7). Using a more sensitive chemiluminescence detection method, however, showed that the antiserum against the intact cytochrome b_5 recognized both the aggregated and the monomeric forms of the protein. Antiserum against the truncated cytochrome b_5 recognised only the truncated protein and not aggregate forms of the intact protein. Immunological studies with the trypsin truncated form of cytochrome b_5 revealed valuable information about the aggregation of cytochrome b_5 . The hydrophobic, membrane-binding region of cytochrome b_5 was clearly essential for the aggregation of the protein. In addition, the hydrophobic membrane binding domain plays an important role in the folding of cytochrome b_5 and it was evident that different domains or regions of the molecule are exposed as epitopes in the truncated form as opposed to those exposed by the full-length native molecule. This is an interesting observation since the truncated cytochrome b_5 exhibits spectral properties identical to the native intact protein.

An antiserum, capable of recognising both the aggregated and monomeric forms of cytochrome b_5 , was prepared by immunising rabbits with purified full length ovine cytochrome b_5 . The IgG fraction recognising only the monomeric form of cytochrome b_5 , was subsequently affinity-purified from the whole serum. Due to the nature of purified cytochrome b_5 in solution, the immobilisation of a homogenous monomeric form of the protein on a matrix was not possible. The monomeric and tetrameric forms of purified ovine cytochrome b_5 was therefore first separated by SDS PAGE and subsequently transferred and immobilised onto a nitrocellulose membrane which served as an affinity matrix. Incubation of whole serum with the immobilised monomer and aggregate forms of cytochrome b_5

allowed the isolation of two types of immunoglobulins — one type recognising the monomeric and the other recognising the tetrameric form of the hemoprotein. Using the immunoglobulin fractions it was shown that ovine cytochrome b_5 exists in a monomeric as well as a tetrameric form, with the tetrameric form prevailing under most physiological conditions. The two aggregation species differed, with respect to their immunogenicity, not only from each but also from the trypsin truncated form of the protein. These results emphasise, once again, the importance of the membrane binding carboxyl terminal of ovine cytochrome b_5 in aggregation, as well as in the tertiary and quaternary structures of the protein.

The final goal of this study was to investigate the influence of purified ovine cytochrome b_5 on ovine adrenal steroidogenesis. Although, an electron transfer protein, cytochrome b_5 can donate the second electron to cytochrome P450 during certain cytochrome P450 dependent hydroxylation reactions, it cannot donate the first electron. It has been shown that the addition of cytochrome b_5 to a cytochrome P450 system may either inhibit, enhance or have no effect on the metabolism of the substrate, an effect which is dependent on the concentration of cytochrome b_5 (13, 14). Since cytochrome P450 plays an important role in steroidogenesis, it is possible that cytochrome b_5 may influence steroidogenesis. It has in fact been shown that cytochrome b_5 enhances the 17,20-lyase activity of cytochrome P450c17 (15). Although the precise action of cytochrome b_5 having a positive modulating effect on cytochrome P450 is unknown, possible models, discussed in detail in Chapter 6, have been proposed. In previous studies we showed that the cytochrome b_5 constitutively expressed in ovine adrenal endoplasmic reticulum is not sufficient to enhance the 17,20 lyase activity of ovine cytochrome P450c17 (16). Ovine adrenal microsomes used in this study contained cytochrome P450 and cytochrome b_5 in a ratio of 5:1. This ratio is in the same order as the ratio previously determined to be present in for bovine and porcine adrenal microsomes (17). The addition of the trypsin truncated ovine cytochrome b_5 to our ovine microsomal assay system showed no change in the metabolism of either progesterone or pregnenolone. The addition of the intact cytochrome b_5 , however, had a definite effect on progesterone metabolism and a significant increase in deoxycorticosterone production was observed after the addition of cytochrome b_5 . This finding correlated well with previous results obtained by Swart *et al.* (18). Cytochrome b_5 did not enhance the lyase activity of cytochrome P450c17 with progesterone as substrate. Using pregnenolone as substrate, addition of cytochrome b_5 showed a definite stimulation of the 17,20-lyase activity of cytochrome P450c17.

Androstenedione formed during this assay is formed through the metabolism of Δ^5 -steroid substrates rather than through the metabolism of Δ^4 -steroids, since no detectable androstenedione was formed when progesterone was used as substrate. It can be concluded from these steroid substrate metabolism assays that the membrane anchoring domain of cytochrome b_5 is important if not essential for the protein to have an influence on cytochrome P450 activity in ovine adrenal steroidogenesis. In addition, ovine cytochrome b_5 has the ability to stimulate only the lyase activity of ovine cytochrome P450c17 when Δ^5 substrates are used.

During this study we proved conclusively that ovine cytochrome b_5 exists as an aggregate in vivo and that this hemoprotein enhances the 17,20 lyase activity of cytochrome P450c17. To answer the question which form is the active, native form of ovine cytochrome b_5 conclusively, it would be necessary to purify sufficient quantities of monomer and aggregate specific antibodies, carry out immunohistochemical studies as well as inhibition studies in microsomal assay systems. The work presented here has paved the way to answering this question by providing methods which yields consistent results and laying the foundation for the preparation of specific anti-monomer and anti-aggregate antibodies.

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ABSTRACT

This study describes the optimisation of isolation and purification procedures for ovine liver microsomal cytochrome b_5 in order to obtain sufficient material for subsequent aggregation and immunological studies. A method utilising PEG for the precipitation of microsomes was used and compared to calcium chloride precipitation of microsomes. PEG precipitated microsomes yielded double the total amount of protein and had almost three times the cytochrome b_5 content of the calcium chloride precipitated microsomes. This method of microsome preparation proved to be robust and less time consuming, making it ideal for small and large scale preparations. Cytochrome b_5 isolated from these microsomes showed two species with apparent molecular masses of $\sim 60,000$ Da and $\sim 16,000$ Da when analysed on SDS PAGE. The higher molecular mass specie could be converted to the lower molecular mass specie by adding increasing amounts of deoxycholate. HPGPC also showed the presence of two species in aqueous solution in the absence of detergents. In aqueous solution, the levels of the high molecular mass specie could be decreased by lowering the ionic strength of the buffer. Only the high molecular mass specie could be observed when the cytochrome b_5 sample was dissolved in PBS prior to HPGPC. ESMS showed only a single specie with a molecular mass of 15,263 Da for the purified protein, indicating that the higher molecular mass specie is a tetrameric aggregate of the protein. The hydrophobic membrane binding domain was removed by means of tryptic digestion in order to establish the role of the carboxyl terminal in ovine cytochrome b_5 aggregation. Due to the low yield of truncated protein obtained by existing methods of tryptic digestion, a novel process was developed to prepare truncated cytochrome b_5 from the intact purified protein with yields ~ 14 times higher than previously reported. ESMS studies showed two species with molecular masses of 10,182 Da and 9,816 Da, the latter being the dominant specie. Antibodies against both the trypsin truncated and intact forms of cytochrome b_5 were subsequently raised to study the aggregation of the protein. Western blot analyses indicated the presence of mainly the tetrameric aggregate in microsomes, raising the question whether the active form of the protein *in vivo* might be the aggregate. A method for successfully preparing monomer and aggregate specific antibodies from the whole serum is described, providing a marker for future immunohistochemical studies into the localisation and aggregation state of cytochrome b_5 *in vivo*. The influence of purified cytochrome b_5 on steroidogenesis in ovine adrenal microsomes was subsequently investigated. The addition of truncated cytochrome b_5 to a microsomal assay system showed no difference from the control sample. Adding intact

cytochrome b_5 , on the other hand, showed a ~100% increase in deoxycorticosterone production with a concurrent decrease of ~60% in 17-OH progesterone levels during progesterone metabolism. This indicated a possible stimulation of cytochrome P450c21 activity with an unchanged cytochrome P450c17 hydroxylase activity. Upon the addition of full length cytochrome b_5 during pregnenolone metabolism, deoxycortisol levels decrease and an increase of ~30% is observed in the androstenedione formation. This lyase activity stimulation reaches its maximum at added cytochrome b_5 :endogenous cytochrome b_5 ratios of 7:1 to 10:1. This study raises the question whether the native form of ovine cytochrome b_5 in adrenal steroidogenesis is a tetramer, while at the same time cautioning against the use of modified recombinant proteins in *in vivo* assay systems.

APPENDIX A

APPENDIX B



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SHEEP ADRENAL CYTOCHROME b_5 : ACTIVE AS A MONOMER OR A TETRAMER IN VIVO?

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ABSTRACT

Cytochrome b_5 (cyt b_5) is an ubiquitous hemoprotein also associated with microsomal cytochromes P450. It has been reported that cyt b_5 influences cytochrome P450-dependent catalyses through electron transport as well as direct protein–protein interactions. To investigate the influence of cyt b_5 on ovine adrenal steroidogenesis, we isolated and characterized cyt b_5 from ovine liver. The molecular mass of the purified protein was 15,260 as determined by electrospray mass spectrometry. SDS-Polyacrylamide gel electrophoresis, even after stringent detergent and mercaptoethanol pre-treatment, indicated multimeric forms of the protein, the most prominent being the tetramer (± 60 kDa) with minor bands corresponding to the monomer (± 16 kDa) and dimer (± 30 kDa). Trypsin treatment of cyt b_5 resulted in a truncated enzyme with a molecular mass of ± 10 kDa. The aggregation of cytochrome b_5 was abolished by the tryptic removal of the membrane binding region. In Western blot analyses antibodies against the truncated protein recognised only this low molecular mass form and not the full length cyt b_5 , or any of the higher molecular complexes, showing the involvement of the membrane binding domain of the protein, not only in aggregation, but also in the quaternary structure which determines epitope presentation for antibody production. Immunoblot analyses of sheep adrenal microsomes with the anti-truncated cyt b_5 antibody were also negative. Immunoblot analyses and immunocytochemistry of adrenal tissue with

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antibodies against the full length cyt b_5 indicated that the tetrameric form of the protein was in all probability the dominant specie in vivo.

INTRODUCTION

Cytochrome b_5 (cyt b_5) is an electron transfer hemoprotein that catalyses a wide range of reactions in mammalian tissue. The protein exists in two forms, an amphipathic membrane-bound form and a water-soluble cytosolic form. The membrane-bound form is found in the endoplasmic reticulum and mitochondria of all cells and consists of a hydrophobic membrane-anchoring domain and a hydrophilic catalytic domain.^[1] During adrenal and gonadal steroidogenesis cyt b_5 influences the action of the cytochromes P450-dependent steroid 17α -hydroxylase and 21 -hydroxylase (CYP17 and CYP21). These enzymes play a central role in glucocorticoid, mineralocorticoid, and androgen biosynthesis and a number of studies have shown the importance of cyt b_5 in C_{19} -steroid production by CYP17.^[2,3]

We have previously shown, in preliminary studies, that cyt b_5 influences the ratio of deoxycorticosterone to deoxycortisol production in ovine adrenal microsomes.^[4] During our previous investigations we encountered, what appeared to be, higher molecular mass aggregates in our cyt b_5 isolates. In this study the apparent aggregation of ovine cyt b_5 was investigated and the question arose whether the protein exists as a monomer or an aggregate in vivo. Previous research has shown that the aggregation of cyt b_5 can be abolished by detergents (deoxycholate) and it was concluded that the endoplasmic reticulum membrane will have the same influence on the enzyme in vivo.^[5] It was, however, also demonstrated by Calabro et al. that low protein and salt concentrations favoured smaller aggregates and that a pure monomeric form of cyt b_5 could be isolated by size-exclusion chromatography under these conditions.^[6] These results indicated that the aggregation of cyt b_5 in vivo was influenced by a number of factors.

To prevent cyt b_5 aggregation in aqueous solution the hydrophobic membrane-anchoring domain can be removed by means of tryptic digestion. In more recent work it was suggested that cyt b_5 influences CYP17 activity, not through direct electron transfer, but by steric interactions. These findings implicate that cyt b_5 would require its hydrophobic membrane binding domain to influence steroidogenesis.^[7] In this study we investigated the aggregation state of ovine cyt b_5 in solution, adrenal microsomes and in situ.

MATERIALS AND METHODS

Isolation of Full Length and Truncated Cytochrome b_5

Ovine liver microsomes were prepared as described by Yang and Cederbaum.^[8] Cytochrome b_5 was isolated from the ovine liver microsomes as

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described by Strittmatter et al.^[9] Truncated cyt b_5 was prepared as follows. Purified cyt b_5 solution (0.5 mL) was dialysed against 10 volumes of a 10 mM *Tris*-acetate buffer (0.1 mM EDTA, pH 8.1) and loaded onto a DEAE-cellulose column (1 × 5 cm) equilibrated with the same buffer. After washing the column with 20 mL of the equilibration buffer, a solution containing approximately 5000 BAEE units/mL trypsin in equilibration buffer was circulated through the column for 180 min at 0.36 mL/min. The column was washed extensively with 50 mL of equilibration buffer to remove all the trypsin and protein fragments. The tryptic fragment was eluted with a 250 mM NaSCN solution in 10 mM *Tris*-acetate (0.1 mM EDTA, pH 8.1). The eluate was subsequently dialyzed against equilibration buffer to remove excess salt.

Electrospray Massspectrometry of Purified Cytochrome b_5

Samples for electrospray massspectrometry (ES-MS) analysis were prepared by dialysing the purified cyt b_5 solution (intact or fragment) overnight against deionised water. The solution was subsequently diluted 1:20 in an 50% acetonitrile solution to a final amount of 0.35 μ mol. Solvent (acetonitrile:water—50:50 v/v) was delivered to the ES-MS using a Pharmacia/LKB2249 HPLC pump at 15 μ L/min. The sample (10 μ L) was injected via a Rheodyne injection valve into the solvent stream and introduced through a conventional electrospray probe with the capillary voltage set at 3.5 kV and the cone voltage at 70 V. The source temperature was 80°C and a coaxial flow of nitrogen gas at 300 NL/h was applied. The instrument was operated and the data processed by the MassLynx software supplied by the manufacturers. The multiple charged ions of cyt b_5 were observed by scanning the first mass analyser of the instrument from m/z = 400–1500 at a scanning speed of 100 amu/second (atomic mass units per second).

Raising of Antibodies Against Full Length and Truncated Cytochrome b_5

Antibodies to full length and truncated cyt b_5 were raised according to the method of Bellstedt et al.^[10]

SDS-PAGE and Western Blot Analyses

SDS-PAGE was carried out according to the method of Laemmli.^[11] Western blot analyses were carried out with the West DuraTM western blotting kit from Pierce Chemical Co., Rockford, Ill, USA.



High Performance Gel Permeation Chromatography

High performance gel permeation chromatography (HPGPC) was done using a Waters HydrogelTM column connected to a Waters HPLC-system equipped with a Waters 991 photo diode array detector at a flowrate of 0.7 mL/min with the buffer systems and conditions as described by Calabro et al.^[6]

Assays for Adrenal Microsomal Steroidogenic Activity

Adrenal microsomal assays were carried out as described by Swart et al.^[4]

RESULTS AND DISCUSSION

The purity of the full length and truncated cyt b₅ was assessed for by means of SDS-PAGE. The full-length protein exhibited a major protein band corresponding to a molecular mass of ± 16 kDa which represented the monomeric form of cyt b₅. Several bands corresponding to multimeric forms of the protein was, however, also observed. Electrospray massspectrometry analyses of the cyt b₅ solution loaded onto the gel indicated a single protein (Mr 15260) (Fig. 1). This result confirmed that SDS-PAGE normally used for the resolution of protein complexes did not fully dissociate the cyt b₅ aggregates. The truncated cyt b₅ showed no aggregation, only a single band corresponding to a molecular mass of ± 10 kDa.

High performance gel permeation chromatography analyses of the purified protein under conditions of low ionic strength (10 mM Tris, 0.1 mM EDTA, pH 8.15) yielded two peaks [Fig. 2(A)]. Both fractions exhibited a typical cyt b₅ absorption spectrum. These two fractions were collected and immediately rechromatographed under the same conditions. Each sample produced a single peak (results not shown). When the same two fractions were incubated for 24 hours before they were rechromatographed, the higher molecular mass component dissociated into two fractions while the lower molecular mass fraction remained a single peak. High performance gel permeation chromatography in phosphate buffered saline (PBS) yielded a single peak which correlated with the higher molecular mass fraction [Fig. 2(B)]. High performance gel permeation chromatography of truncated cyt b₅ yielded a single peak at a lower molecular weight than any of the other sample chromatographed. The retention time of this peak remained the same irrespective of the salt or protein concentration [Fig. 2(C)]. These results showed that, like rabbit liver cyt b₅, the aggregation of ovine cyt b₅ was influenced by ionic strength. In addition the aggregated form of the enzyme could be stabilized under appropriate conditions of ionic strength and pH.

The anti-serum against the intact purified cyt b₅ recognised the monomeric, trimeric, and tetrameric species (Fig. 3) of the enzyme. In the microsomal preparations of ovine adrenal, liver and testes and bovine adrenal the tetrameric

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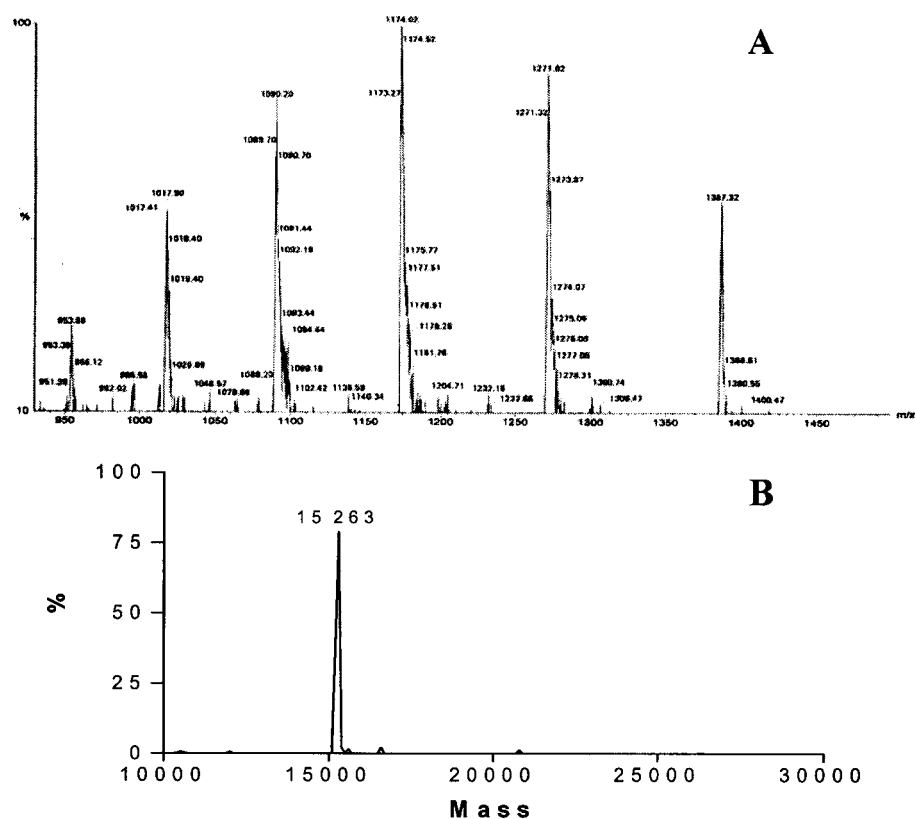


Figure 1. (A) Electrospray mass spectrum of purified ovine cyt b_5 . Each peak represents the same protein with a different charge to mass ratio. (B) Deconvolution of the ESMS spectrum was carried out by MaxEnt program[®]. The molecular mass indicated for cyt b_5 was 15,263. No other proteins could be detected in the sample.

aggregate predominated. The same anti-serum recognised cyt b_5 in the zona fasciculata of the ovine adrenal cortex as well as the adrenal medulla (results not shown). Antibodies raised against the truncated protein did not show immunoprecipitation in any of the above-mentioned microsomal preparations.

The influence of cyt b_5 on adrenal microsomal progesterone metabolism is shown in Fig. 4. The truncated form of the protein did not have any influence on the conversion of progesterone to deoxycortisol and deoxycorticosterone. Addition of full-length cyt b_5 increased the production of deoxycorticosterone with a concomitant reduction in deoxycortisol production. These results are in accordance with results previously published.^[4]

At physiological ionic strength and pH ovine cyt b_5 exists as a tetramer with a Mr of ± 60 kDa. Immunoblot analyses of ovine and bovine steroidogenic microsomes indicated the tetrameric form as the predominant specie. Although the antibody could detect the monomeric form of the purified protein no

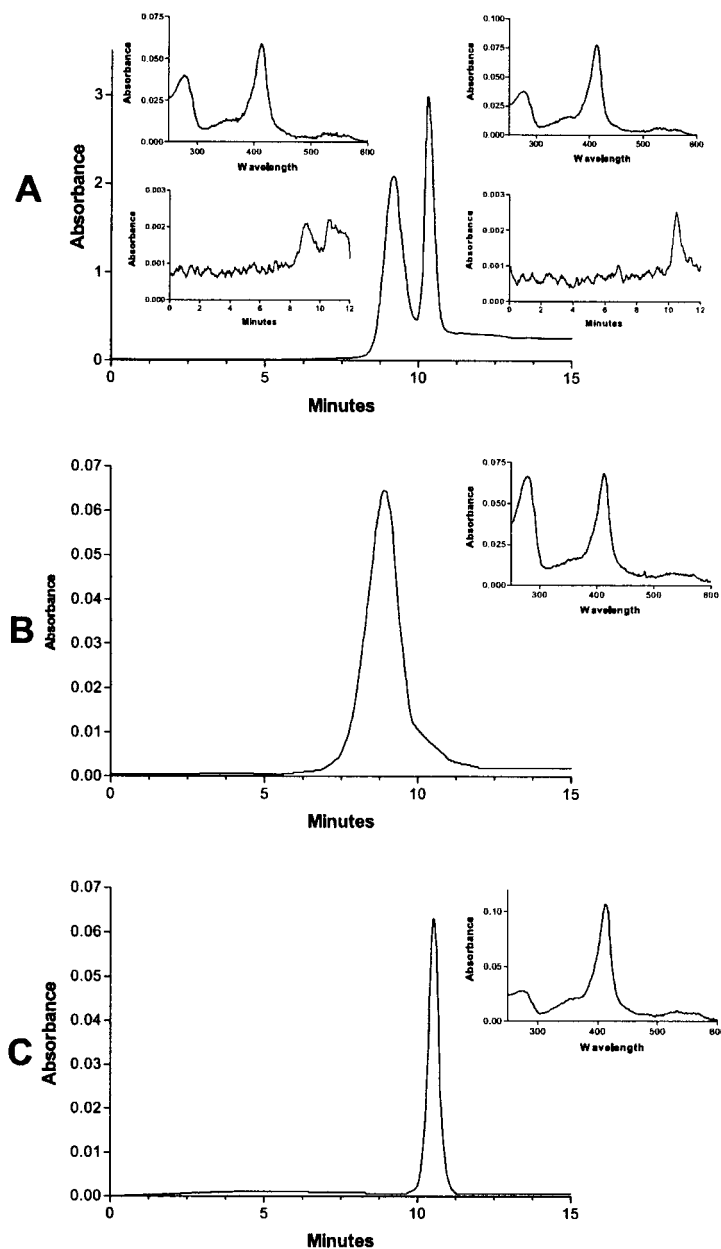


Figure 2. High performance gel permeation chromatogram of cyt b_5 in a low ionic strength buffer (10 mM Tris, 0.1 mM EDTA, pH 8.15) (A). Two forms of cyt b_5 with different retention times was observed. Both exhibited a typical cyt b_5 absorption spectrum. When rechromatographed after a 24-hour incubation period, the fraction correlating with the monomer still exhibited a single peak while the fraction that correlated with the aggregate again exhibited two peaks. When the buffer was changed to phosphate buffered saline, only a single peak, with the same retention time as the aggregate, was observed (B). Irrespective of the salt or protein concentration, the truncated cyt b_5 always exhibited a single peak (C).

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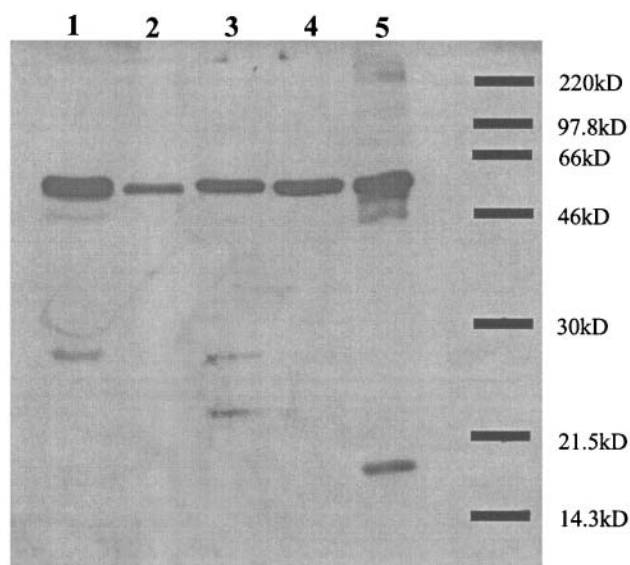


Figure 3. Western blot analyses of: lane 1; ovine liver microsomes, lane 2; bovine adrenal microsomes, lane 3; ovine adrenal microsomes, lane 4; ovine testicular microsomes, lane 5; purified cyt b_5 . Into wells 1–4, 50 μ g of total protein was loaded, while 10 μ g of purified cyt b_5 was loaded into well 5. Polyclonal rabbit anti-cyt b_5 was used at a dilution of 1 : 2000.

immunoprecipitation was detected at 16 kDa in Western blot analyses of adrenal or liver microsomes. These results indicate that under conditions of physiological pH and ionic strength the enzyme is in a tetrameric form in the endoplasmic membrane. These findings are in contrast with previously published results of Doeblner et al.^[12]

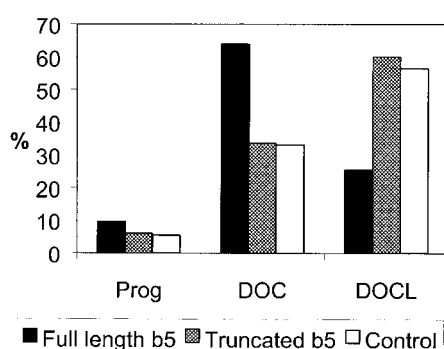


Figure 4. Progesterone metabolism in ovine adrenal microsomes ([cytochrome P450] = 0.09 μ M). Progesterone, deoxycortisol (DOC), and deoxycorticosterone (DOCL) are indicated after a 14-min incubation period. No cyt b_5 was added to the control which had a [Cytochrome P450]:[cyt b_5] = 2.26. The addition of truncated cyt b_5 had no effect on the progesterone metabolism. Addition of full-length cyt b_5 (1 μ M) increased the production of DOC and concomitantly reduced DOCL production.



which stated that neither cyt b_5 nor its membrane-binding domain will self associate in phosphatidylcholine vesicles. These experiments were, however, carried out in an artificial system that contained no other proteins except cyt b_5 . In addition, a purified recombinant mutant cyt b_5 was used in these studies. Although the aggregation state of intact cyt b_5 in adrenal microsomes cannot be readily determined, the addition of the aggregate to adrenal microsomes significantly influenced the metabolism of progesterone while the truncated form of cyt b_5 had no effect. The aggregation of cyt b_5 in the endoplasmic membrane and the influence of the aggregation on the biological activity of the enzyme will be further investigated.

ACKNOWLEDGMENTS

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Ovine steroid 17 α -hydroxylase cytochrome P450: characteristics of the hydroxylase and lyase activities of the adrenal cortex enzyme

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Abstract

The steroid 17-hydroxylase cytochrome P450 (CYP17) found in mammalian adrenal and gonadal tissues typically exhibits not only steroid 17-hydroxylase activity but also C-17,20-lyase activity. These two reactions, catalyzed by CYP17, allow for the biosynthesis of the glucocorticoids in the adrenal cortex, as a result of the 17-hydroxylase activity, and for the biosynthesis of androgenic C₁₉ steroids in the adrenal cortex and gonads as a result of the additional lyase activity. A major difference between species with regard to adrenal steroidogenesis resides in the lyase activity of CYP17 toward the hydroxylated intermediates and in the fact that the secretion of C₁₉ steroids takes place, in some species, exclusively in the gonads. Ovine CYP17 expressed in HEK 293 cells converts progesterone to 17-hydroxyprogesterone and pregnenolone to dehydroepiandrosterone via 17-hydroxypregnenolone. In ovine adrenal microsomes, minimal if any lyase activity was observed toward either progesterone or pregnenolone. Others have demonstrated the involvement of cytochrome *b*₅ in the augmentation of CYP17 lyase activity. Although the presence of cytochrome *b*₅ in ovine adrenocortical microsomes was established, ovine adrenal microsomes did not convert pregnenolone or 17-hydroxypregnenolone to dehydroepiandrosterone. Furthermore the addition of purified ovine cytochrome *b*₅ to ovine adrenal microsomes did not promote lyase activity. We conclude that, in the ovine adrenal cortex, factors other than cytochrome *b*₅ influence the lyase activity of ovine CYP17.

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The abilities to accept more than one substrate and to produce multiple products from single substrates place the adrenal and gonadal steroidogenic enzymes in a rare position in mammalian metabolism. The steroidogenic enzymes most notable for multiple substrate and product catalyses are steroid 17 α -hydroxylase cytochrome P450 (CYP17)¹ and 3 β -hydroxysteroid dehydrogenase/ $\Delta^5 \rightarrow 4$ -isomerase (3 β -HSD). The latter has the ability to

convert the 3 β -hydroxy- Δ^5 -steroid precursors pregnenolone (P5) and 17-hydroxypregnenolone (17-OHP5) and dehydroepiandrosterone (DHEA) to the corresponding Δ^4 3-ketosteroids, while CYP17 can, depending on the species and the tissue, convert P5 and progesterone (P4) to the corresponding 17-hydroxy derivatives as well as the two sex steroid precursors, DHEA and androstenedione (A4).

Because of the competition between CYP17 and 3 β -HSD for the same substrates, the ratio of one enzyme activity to the other, as well as substrate specificities, vitally determine the steroidogenic pathway and catalytic efficiency with which steroid intermediates and end products are formed. Furthermore, as only a single CYP17 gene has been demonstrated in any species, variations in the ratio of 17 α -hydroxylase to 17,20-lyase activity, seen in various adrenal and gonadal tissues,

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¹ Abbreviations used: 3 β -HSD, 3 β -hydroxysteroid dehydrogenase/ $\Delta^5 \rightarrow 4$ -isomerase; A4, androstenedione; DHEA dehydroepiandrosterone; CYP17; steroid 17 α -hydroxylase cytochrome P450; P5, pregnenolone; P4, progesterone; 17-OHP5, 17-hydroxypregnenolone; 17-OHP4, 17-hydroxyprogesterone; 4MA, 17 β -N,N-diethylcarbomyl-4-aza-4-methyl-5 α -androstane-3-one; ES-MS, electrospray mass spectrometry.

indicate that these two activities of CYP17 are modulated on a posttranscriptional level [1,2]. Testicular CYP17, for instance, has a relatively higher 17,20-lyase activity compared to 17-hydroxylase than adrenal CYP17 and much of the 17-hydroxy C₂₁-steroid intermediates are quantitatively converted to C₁₉ steroids in the testes. The 17 α -hydroxylation of P5 or P4 takes place wherever CYP17 is expressed but the subsequent lyase activity for the hydroxylated intermediates differs markedly. Rat and porcine CYP17 metabolize both 17-OHP5 and 17-hydroxyprogesterone (17-OHP4) to DHEA and A4, respectively [3,4]. Human and bovine CYP17 are unable to convert 17-OHP4 to A4 [5,6], while guinea pig CYP17 cannot convert 17-OHP5 to DHEA but does metabolize 17-OHP4 to A4 [7].

It is evident that the relative natures of 17 α -hydroxylase and 17,20-lyase activities are, by and large, species-specific and are also influenced by a number of other factors. The regulation of the relative flow of steroids through the glucocorticoid and adrenal androgen pathways is unclear. It is established that the human adrenal cortex acquires a marked capacity for adrenal androgen biosynthesis at the time of adrenarche, probably developmentally regulated by changes in the function of the zona reticularis. Electron transfer proteins, such as NADPH cytochrome P450 reductase, cytochrome 21-hydroxylase (CYP21), and 3 β -HSD [4,8,9], as well as the liposomal membranous environment [10], are factors which appear to influence the lyase activity and therefore adrenal androgen production.

The production of C₁₉ steroids in gonads and importantly ovine placenta at the time of parturition requires the manifestation of CYP17 lyase activity [11]. The purpose of this study was to compare the activity of ovine CYP17 expressed in HEK 293T cells with the activity of the enzyme in ovine adrenal microsomes. The lyase activity of CYP17 in ovine adrenal microsomes toward the Δ^5 steroids, P5 and 17-OHP5, was also evaluated.

Materials and methods

Preparation of ovine adrenal microsomes

The adrenals of sheep were immediately removed after slaughter at local abattoirs and stored on ice. All subsequent procedures were performed at 4°C. After removal of the capsule and medulla, the adrenals were homogenized in three parts of 10 mM Tris–HCl buffer (pH 7.4) containing 1.0 mM EDTA and 0.25 M sucrose and centrifuged at 500g for 10 min. The supernatant fraction was subsequently centrifuged at 11,000g for 16 min, and the postmitochondrial fraction was collected. To prepare the microsomes a 50% (w/v) PEG 8000 solution was added slowly with stirring to the

postmitochondrial supernatant to a final concentration of 8.5% and the mixture was stirred for 10 min at 4°C and then centrifuged at 13,000g for 20 min. The microsomal pellets were suspended in 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM KCl and 1.0 mM EDTA. PEG was again added to the suspension to a final concentration of 8.5% and the mixture was stirred and centrifuged at 13,000g for 20 min. This procedure was repeated twice until the supernatant was clear. The final microsomal pellet was suspended in 10 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA and stored at –70°C until use.

Cytochrome P450 and cytochrome b₅ assays

The cytochrome P450 and cytochrome b₅ contents of microsomal preparations were determined as previously described [12,13].

Steroid-induced difference spectra

The microsomal preparation (1 mg protein/ml, 0.45 nmol P450/mg protein) was divided into two optically matched cuvettes and a baseline was recorded between 385 and 500 nm using a Kontron Uviko 860 spectrophotometer. Steroid dissolved in ethanol was subsequently added to the sample cuvette and an equal volume of ethanol to the reference cuvette. The contents of both cuvettes were mixed by gentle inversion and a difference spectrum was recorded between 385 and 500 nm.

Cloning of ovine CYP17

Total RNA was extracted from ovine adrenal cortex with guanidinium thiocyanate followed by centrifugation in a cesium chloride solution [14]. Polyadenylated RNA was isolated using a mRNA Capture Kit (Boehringer–Mannheim Biochemicals).

Complementary cDNA was synthesized by reverse transcription of mRNA using the Titan One Tube RT-PCR system (Boehringer–Mannheim Biochemicals). The reverse transcription reaction was performed at 50°C for 30 min after which thermocycling was carried out directly. Ovine-specific primers complementary to the 5' (sense) and 3' (antisense) termini of ovine CYP17, 5'-aagcttgacacaatgtgggtgctc-3' (includes a *Hind*III restriction site) and 5'-tctcgagtcagtgaggtgctaccctca-3' (includes a *Xho*I restriction site), respectively, were derived from GenBank Accession No. L40335 for ovine CYP17. The RT-PCR product was gel purified, digested with *Hind*III and *Xho*I, and cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Paisley, UK). Nucleotide sequences of both strands, purified RT-PCR product and cloned cDNA, were determined using the BigDye Version 2 terminator sequencing kit (Model

373A ABI; Applied Biosystems, Foster City, CA, USA). These were in accord with that sequence except that amino acid residue 434 was Asn (caa) as noted in updated GenBank Accession No. AF251388 (recorded in L40355 erroneously as cta for Tyr).

Expression of sheep CYP17 in HEK 293T cells

HEK 293T cells were plated into six-well tissue culture dishes at a density of 250,000 cells/well. The cells were cultured for 24 h and transfection with the pcDNA3.1 vector was achieved using the GenePORTER II (Gene Therapy Systems, San Diego, CA, USA) transfection protocol.

Assay for microsomal steroid metabolism

Incubations were performed in a shaking water bath at 37°C in a total volume of 1 ml. The reaction was carried out in a 50 mM Tris buffer solution (pH 7.4) that contained 1% bovine serum albumin and 50 mM NaCl. In addition, the reaction mixture contained microsomal preparation (0.35 μ M P450), NADPH (1 mM), MgCl₂ (10 mM), isocitrate (2 mg/ml), and isocitrate dehydrogenase (0.2 U/ml). After a 5-min preincubation period, the reaction was initiated by the addition of the appropriate steroid substrate in ethanol. The steroids present in the reaction mixtures were extracted from the incubation mixture with methylene chloride (9:1 solvent to incubation mixture). The water phase was removed and the methylene chloride was evaporated under a stream of air or nitrogen. The residues were subsequently dissolved in the appropriate solvent for TLC or HPLC analyses. Steroid recovery was greater than 85%.

HPLC analyses of steroid mixtures

For radioactive and Δ^4 steroids, methanol (100 μ l) was added to the dried steroid residue and the resultant solution subjected to HPLC analysis on a Novapak C₁₈ column (see legends to figures for solvents and detection methods). Before HPLC analyses and UV-detection, Δ^5 steroids were converted to Δ^4 steroids by cholesterol oxidase treatment. Cholesterol oxidase (2 U/ml in 1% sodium deoxycholate, pH 7.4) was added to the dried steroid extracts. The conversion to the 4-ene-3-one derivatives was performed at 37°C for 20 min. Steroids were subsequently extracted as described above and analyzed by HPLC.

TLC analyses of steroid mixtures

Thin-layer chromatography was carried out on aluminum TLC plates coated with Silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany). The solvent system used for development was chloroform:ethyl acetate 4:1

(vol/vol). Identification and quantification of steroids was with a TLC radioimager (Bioscan 200 Imaging Scanner; Bioscan Imaging Systems, Washington DC).

Isolation and purification of ovine cytochrome b₅

Sheep liver was obtained from the abattoir and kept on ice until used. Liver microsomes were prepared and fractionated as described by Yang and Cederbaum [15]. All procedures were carried out at 4°C. The supernatant of the fractionation step was loaded on a DEAE-cellulose column (3.2 \times 20 cm) equilibrated with 80 mM Tris acetate, 1 mM EDTA, 2% Triton X-100, pH 8.1. The column was washed with 3 bed volumes of equilibration buffer before cytochrome b₅ reductase was eluted as a yellow band with 0.1 M Tris acetate, 1 mM EDTA, 2% Triton X-100, pH 8.1. After elution of the reductase, a linear gradient from 0 to 50 mM NaSCN was run over 3 bed volumes in 10 mM Tris acetate, 1 mM EDTA, 0.1% deoxycholate, 0.2% Triton X-100, pH 8.1. The cytochrome b₅ was subsequently eluted with 90 mM NaSCN in the 10 mM Tris acetate buffer. This fraction was dialyzed overnight against at least ten volumes of 10 mM Tris acetate buffer and applied to a DEAE-cellulose column (2 \times 20 cm) equilibrated with 10 mM Tris acetate, 1 mM EDTA, 0.2% Triton X-100, 0.05% deoxycholate, pH 8.1. After the column was washed with 3 bed volumes of equilibration buffer, a linear gradient of NaSCN, in equilibration buffer, was run over 3 bed volumes from 0 to 50 mM NaSCN to remove trace impurities. The final cytochrome b₅ preparation was obtained by running a linear NaSCN gradient from 50 to 90 mM NaSCN over 5 bed volumes. The solution containing purified cytochrome b₅ was subsequently dialyzed overnight against 10 volumes of elution buffer (10 mM Tris acetate, 0.1 mM EDTA, pH 8.1) and the resulting dialysate was applied to a 1 \times 5-cm DEAE-cellulose column equilibrated with elution buffer. The column was washed with 2 L of elution buffer to remove the Triton X-100. The cytochrome b₅ was eluted with Tris buffer (10 mM Tris acetate, 0.1 mM EDTA, 0.25 M NaSCN, 0.25% sodium deoxycholate, pH 8.1) and the eluate was dialyzed overnight against elution buffer. The resulting cytochrome b₅ preparation was stored at –80°C.

Preparation of antibodies against cytochrome b₅

Polyclonal antibodies against the purified cytochrome b₅ were raised in rabbits as previously described [16,17].

Western blot analyses

Purified cytochrome b₅ and microsomal preparations from ovine liver, testes, and adrenals and bovine liver microsomes were subjected to SDS-PAGE according to

the method of Laemmli using typical conditions for the denaturing of membrane-bound proteins [18]. Western blot analyses were carried out with rabbit anti-sheep cytochrome b_5 IgG at a 1:2000 dilution using the SuperSignal West Dura Western Blotting kit (Pierce-Chemical, Rockford, IL, USA).

Electrospray mass spectrometry of purified cytochrome b_5

Samples for ES-MS analysis were prepared by dialyzing the purified cytochrome b_5 solution (intact or fragment) overnight against deionized water. The solution was then diluted 1:20 in a 50% acetonitrile solution to a final amount of 0.35 μmol . Solvent (acetonitrile:water—50:50 v/v) was delivered to the ES-MS using a Pharmacia/LKB2249 HPLC pump at 20 $\mu\text{L}/\text{min}$. The sample (10 μL) was injected via a Rheodyne injection valve into the solvent stream and introduced through a conventional electrospray probe with the capillary voltage set at 3.5 kV and the cone voltage at 70 V. The source temperature was 80 $^{\circ}\text{C}$ and a coaxial flow of nitrogen gas at 100 psi was applied. The instrument was operated and the data were processed by the MassLynx software supplied by the manufacturers. The multiple charged ions of cytochrome b_5 were observed by scanning the first mass analyzer of the instrument from $m/z = 400$ –1500 at a scanning speed of 100 amu/s.

Results

Cytochrome P450 and cytochrome b_5 concentrations

A CO-induced difference spectrum of reduced versus oxidized ovine adrenal microsomal cytochrome P450 is shown in Fig. 1A and the NADH reduced versus oxidized difference spectrum of cytochrome b_5 in the same preparation is shown in Fig. 1B. The cytochrome P450 concentration in sheep adrenal microsomes was 0.45 nmol/mg protein (actually computed from dithionite-reduced plus CO versus dithionite-reduced differ-

ence spectra) and the cytochrome b_5 content was 0.2 nmol/mg protein.

Cytochrome P450 substrate-induced difference spectra

The ovine adrenal microsomal cytochrome P450 substrate-induced difference spectra are shown in Fig. 2. All four steroids, P4, P5, and their respective 17-hydroxysteroid derivatives, bound to the microsomal cytochrome P450 inducing typical type I spectra with an absorption maximum at 385 nm and an absorption minimum at 420 nm.

Progesterone and pregnenolone metabolism by ovine adrenal microsomes

The conversion of P4 to 17-OHP4 and subsequently to deoxycortisol by sheep adrenal microsomes is shown in Fig. 3. During the 12-min incubation period approximately 50% of the total P4 added was converted to deoxycorticosterone and approximately 20% to deoxycortisol. No formation of A4 was detected. [^3H]Pregnenolone was converted to 17-OHP5, P4, deoxycortisol, and deoxycorticosterone by sheep adrenal microsomes (Fig. 4). After the 15-min incubation period no 17-OHP4, androstenedione, or DHEA could be detected. The influence of 3β -HSD on microsomal P5 metabolism was subsequently minimized by the addition of the potent 3β -HSD inhibitor, 17 β -*N,N*-diethylcarbomyl-4-aza-4-methyl-5 α -androstane-3-one (4MA). In the presence of the inhibitor only P5 and 17-OHP5 could be identified

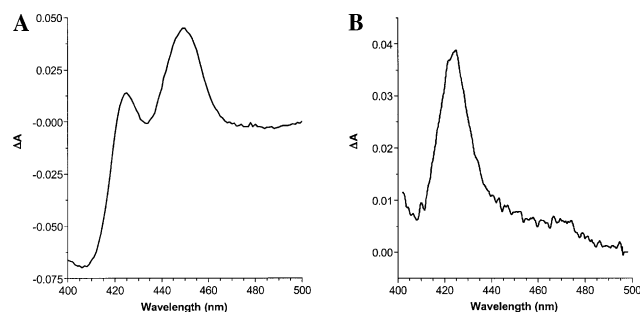


Fig. 1. (A) CO-induced reduced versus oxidized difference spectrum of ovine adrenal microsomal cytochrome P450. (B) NADH reduced versus oxidized difference spectrum of ovine adrenal microsomes.

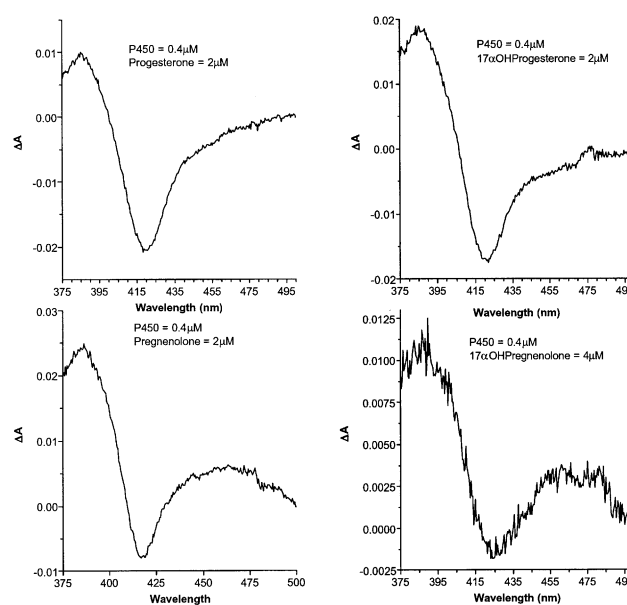


Fig. 2. Type I induced difference spectra of ovine adrenal microsomal cytochrome P450 produced with P4, P5, and their 17-hydroxy derivatives.

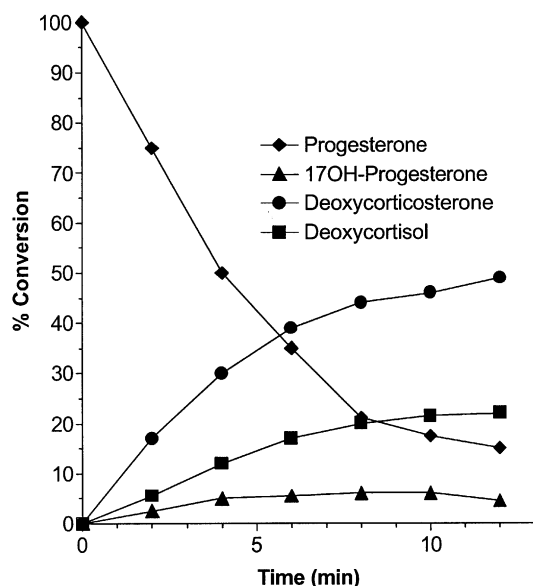


Fig. 3. Metabolism of P4 (20 μ M) by ovine adrenal microsomes. Steroids were separated and quantified by HPLC using a Novapak C₁₈ column with mobile phases consisting of solvent A (water/methanol 40/60) and solvent B (methanol). The column was eluted for 15 min with solvent A followed by a linear gradient from 100% A to 100% B in 10 min and a further 10-min isocratic elution with solvent B. Tritiated steroids were detected with a Radiomatic A-100 flow detector. [P450] 0.35 μ M, [³H]progesterone 100,000 cpm/ml.

in the incubation mixture and no DHEA was formed (Fig. 5A). When 17-OHP5 was provided as substrate, no other steroids except the 17-OHP5 substrate could be detected in the reaction mixture after a 30-min incubation period, indicating that this steroid was not readily metabolized in sheep adrenal microsomes (Fig. 5B). A time course of the metabolism of P5 to 17-OHP5 by ovine adrenal microsomes in the presence of 4MA is given in Fig. 6.

When an ovine adrenal microsomal assay (0.35 μ M P450) was supplemented with 0.5 μ M purified ovine liver cytochrome *b*₅ (i.e., a fourfold increase in cytochrome *b*₅ concentration) CYP17-related activities were not increased compared to control activities as determined over a 10-min assay period; in particular, no 17-OHP5 C-17,20-lyase activity was observed.

Metabolism of progesterone and pregnenolone in HEK 293T cells expressing ovine CYP17 cDNA

Sheep CYP17 expressed in HEK 293T cells converted P4 to 17-OHP4 as the main metabolite. After an incubation period of 8 h 90% of the added P4 was converted to 17-OHP4 but no A4 was detected. After an incubation period of 12 h, approximately 14% of the radioactivity was associated with A4 (Fig. 7A). In contrast to P4, 70% of the P5 added was converted to the C₁₉ steroid, DHEA, via 17-OHP5 in 12 h (Fig. 7B).

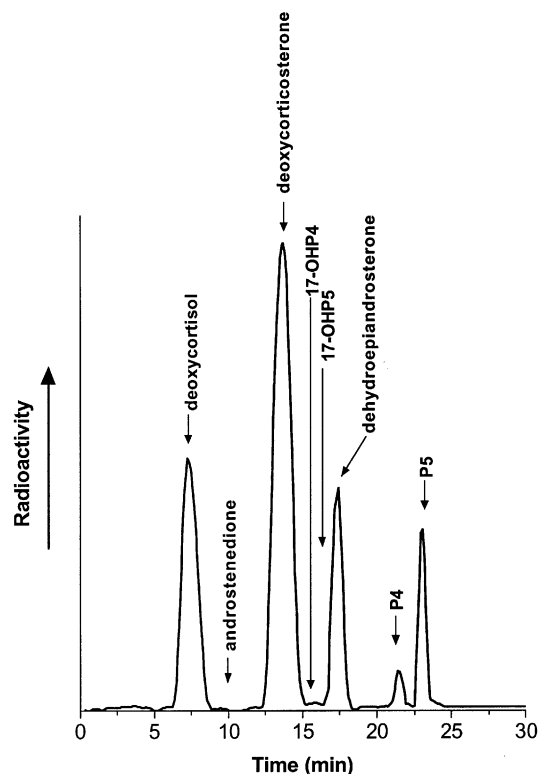


Fig. 4. HPLC chromatogram of P5 metabolites produced by ovine adrenal microsomes. No 17-OHP4 or C₁₉ steroids were detected after a 15-min incubation period. Steroids were separated and quantified by HPLC using a Novapak C₁₈ column with mobile phases consisting of solvent A (water/methanol 50/50) and solvent B (methanol). The column was eluted for 25 min with solvent A followed by a linear gradient from 100% A to 100% B in 5 min and a further 10-min isocratic elution with solvent B. Steroids were detected with a UV detector at 254 nm. [P450] 0.35 μ M, [P5] 20 μ M, [³H]pregnenolone 100,000 cpm/ml. Peaks 1, P5; 2, P4; 3, 17-OHP5; 4, DHEA; 5, 17-OHP4; 6, deoxycorticosterone; 7, A4; 8, deoxycortisol.

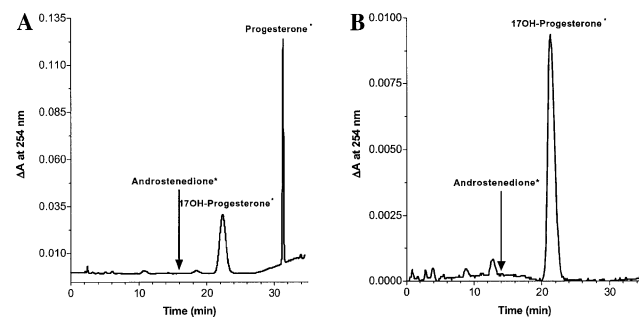


Fig. 5. (A) HPLC chromatogram of P5 metabolites after incubation with ovine adrenal microsomes in the presence of the 3 β -HSD inhibitor 4MA (20 μ M). (B) HPLC chromatogram of 17-OHP5 metabolites after incubation with ovine adrenal microsomes in the presence of the 3 β -HSD inhibitor 4MA (20 μ M). Note. The Δ^5 -3 β -hydroxysteroids were extracted and enzymatically converted to the corresponding Δ^4 steroids by cholesterol oxidase treatment (0.2 mU/ml) before HPLC separation. [P450] 0.35 μ M, [17-OHP5] and [P5] 30 μ M.

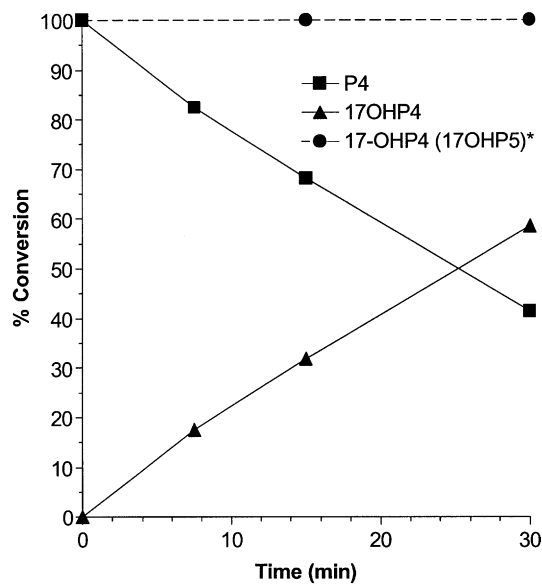


Fig. 6. Time course of P5 and 17-OHP5 metabolism by ovine adrenal microsomes in the presence of the 3 β -HSD inhibitor 4MA (20 μ M). The dashed line denotes levels of 17-OHP4 derived from 17-OHP5 when the latter was added as the sole substrate. *Note.* The Δ^5 -3 β -hydroxysteroids were extracted and enzymatically converted to the corresponding Δ^4 steroids by cholesterol oxidase treatment (0.2 mU/ml) before HPLC separation. [P450] 0.35 μ M and [P5] 30 μ M.

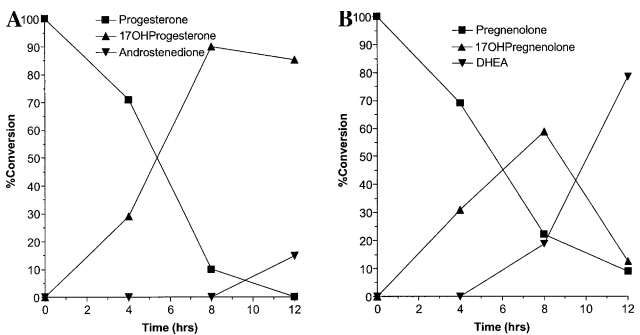


Fig. 7. (A) Time course for the metabolism of P4 by HEK 293T cells transfected with ovine CYP17. (B) Time course for the metabolism of P5 by HEK 293T cells transfected with ovine CYP17. Steroids were separated and quantified by TLC as described under Materials and methods.

Isolation and purification of ovine cytochrome *b*₅

Table 1 presents the protein purification data for ovine cytochrome *b*₅. A 37-fold purification was ob-

Table 1
Protein purification table for the isolation of ovine cytochrome *b*₅ from liver microsomes

Fraction	Total volume (ml)	[Total protein] (mg/ml)	Total protein (mg)	[Cytochrome <i>b</i> ₅] (μ M)	Total cytochrome <i>b</i> ₅ (nmol)	Specific content (nmol/mg)	Purification (fold)	Yield (%)
Microsomes	50	32.48	1624.00					
Triton X-100 extract	430	3.42	1470.60	1.76	750	0.5		100
First DEAE eluate	170	2.52	428.74	3.89	660	1.5	3.00	87.6
Second DEAE eluate	30	0.58	17.46	10.94	330	18.8	36.61	43.5

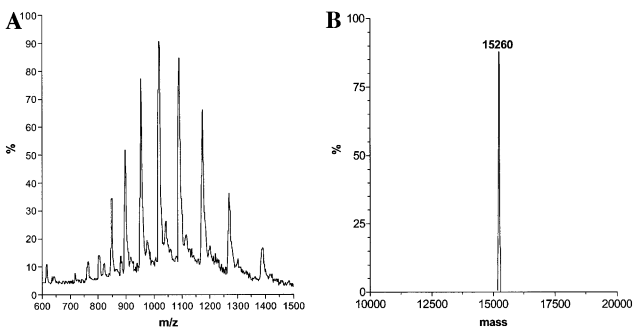


Fig. 8. (A) Electrospray mass spectrum of purified ovine cytochrome *b*₅. The peaks at 803.70, 849.04, 898.85, 964.64, 1017.96, 1090.83, 1174.55, 1272.61, and 1387.86 represent the same protein with different charges. (B) Deconvoluted spectrum of the multiply charged ion spectrum (A). Deconvolution was carried out using the MaxEnt program which is an integral part of the instrument software. The resultant molecular mass of the protein was 15,260 Da.

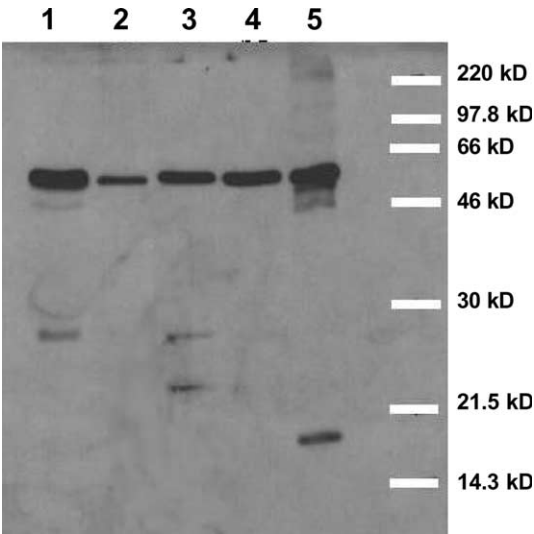


Fig. 9. Immunoblot analyses of microsomes of ovine liver (1), bovine adrenal (2), ovine adrenal (3), ovine testes (4), and purified ovine cytochrome *b*₅ (5). For each of the microsomal preparations, 50 μ g of protein was loaded per lane and for the purified cytochrome *b*₅ preparation, 10 μ g of protein. Rabbit anti-cytochrome *b*₅ serum was used at a 1:2000 dilution.

tained with a cytochrome *b*₅ yield of 44%. ES-MS analyses of the purified cytochrome *b*₅ preparation indicated a single protein with a molecular mass of 15,260 Da (Figs. 8A and B).

Immunoblot analyses

The immunoblot analyses of microsomal preparations from ovine liver, adrenal, and testes and bovine adrenal and of purified sheep cytochrome *b*₅ are shown in Fig. 9. The purified cytochrome *b*₅ gave immunoprecipitation bands at 60 and 15 kDa. The band at 60 kDa is a tetramer of the 15 kDa protein monomer and not an impurity as the ESMS-analyses of this preparation showed that only the 15 kDa species was present (see Figs. 8A and B).

Discussion

Extragenital C₁₉ steroids are secreted in significant amounts only in man and higher primates exhibiting adrenal CYP17 expression. In the adrenals of humans, higher primates, and cattle the zona reticularis, like the gonads, expresses CYP17 and 3 β -HSD although the latter may be quite low in the zona reticularis of non-stressed humans [9]. Therefore, even though the adrenals and gonads are functionally distinct they are both capable of synthesizing similar steroid intermediates. However, P4 is not metabolized to A4 due to the relatively inefficient use of 17-OHP4 as a substrate for 17, 20-lyase activity. A4 in man and bovine is normally biosynthesized via the Δ^5 pathway. Porcine adrenal CYP17 catalyzes the formation of A4 via the Δ^5 and Δ^4 pathways. In contrast in rats, mice, and rabbits, CYP17 expression is restricted to the gonads where A4 is produced by either of the two pathways.

Expression of ovine CYP17 in HEK 293T cells showed that the enzyme has the ability to convert 17-OHP5 to yield DHEA while C₁₉ steroid production from P4 was minimal. This is not surprising as steroid metabolism in ovine testes and placentomes produces the androgen precursors for testosterone and estrogen production [11]. Ovine adrenal microsomes, however, did not produce androgens through either the Δ^5 or the Δ^4 pathway. No A4 was synthesized with P4 as substrate, while P5 metabolism, in the absence of a 3 β -HSD inhibitor, also yielded the precursors for glucocorticoid and mineralocorticoid production in ovine adrenal microsomes. When the actions of 3 β -HSD were arrested by the addition of 4MA, 17-OHP5 was produced as the sole product. Substrate-induced difference spectra of ovine adrenal microsomal cytochrome P450 showed that both P4 and P5, as well as their 17-hydroxy derivatives, had the ability to induce typical type I difference spectra. The interaction of P5 and 17-OHP5, substrates for CYP17 alone, is significant in this regard and indicates that the microsomal enzyme has the ability to bind both substrates and is therefore capable of potentially yielding DHEA, yet this C₁₉ steroid is not produced in adrenal microsomes. It is also notable that ovine adrenocortical

cells, even after prolonged ACTH treatment, did not produce C₁₉ steroids [19]. Normal gonadal and placental androgen production, steroid C-17,20-lyase activity toward 17-OHP5 in HEK 293T cells expressing ovine CYP17, the conversion of P5 as well as P4 to glucocorticoids and mineralocorticoid precursors, and substrate-induced difference spectra together confirm that ovine CYP17 is a fully functional enzyme capable of converting P5 or 17-OHP5 to DHEA. The inability of the adrenal enzyme to produce DHEA in ovine adrenals therefore has to be attributed to one (or more) factor(s) present in the adrenal cortex that specifically affects the formation of DHEA from its Δ^5 precursors. A number of previous studies have shown that cytochrome *b*₅ has the ability to amplify greatly the lyase activity of CYP17 not only in humans [6,20] but also in other species [1,2,21]. As shown in Figs. 1A and B, cytochrome *b*₅ is present in ovine adrenal microsomes at a concentration of 0.2 nmol/mg protein. The ratio of cytochrome P450:cytochrome *b*₅ in adrenal microsomes is about 2.5:1 and, making a reasonable assumption that both CYP17 and CYP21 contribute similarly to total adrenal microsomal P450, the relative level of cytochrome *b*₅ to CYP17 compares well with figures previously reported for ovine gonadal tissue in which adequate Δ^5 -lyase activity is demonstrable [22]. The immunoblot analysis of ovine adrenal microsomes after SDS-PAGE shows that cytochrome *b*₅ is present in an amount comparable to those in microsomes of ovine testes and liver and bovine adrenal tissues. The maintenance of a tetrameric state after the reasonably standard denaturing conditions [18] employed suggests significant secondary structural interactions in this cytochrome structure. In addition the signal for cytochrome *b*₅ in the ovine tissue is on the same order of intensity and character as that for bovine adrenal microsomal cytochrome *b*₅. The typical quaternary structure of cytochrome *b*₅ does not appear to have been addressed in the literature with any detail although multimeric species of human cytochrome *b*₅ have been noted [8]. It remains a possibility that only monomeric cytochrome *b*₅ can effectively promote ovine adrenal CYP17-dependent lyase activity; however, it must be noted that monomeric cytochrome *b*₅ is a very minor species not only in other ovine tissues but also in bovine adrenal where C-17,20-lyase activity is well established. The quaternary structural status of cytochrome *b*₅, or even that of cytochrome P450, under reconstituted activity conditions has not been addressed in the numerous studies examining the effect of cytochrome *b*₅ on cytochrome P450-dependent activities and therefore is an avenue for future studies.

From the results presented in this paper it is evident that ovine adrenal microsomes do not convert P5 or P4 to C₁₉-androgen precursors. The absence in the ovine adrenal cortex of CYP17 lyase activity toward Δ^5 steroids is not because of a lack of cytochrome *b*₅ in ovine

adrenal endoplasmic reticulum. It is therefore deduced that the inability of ovine adrenal CYP17 to lyse 17-OHP5 is due to the presence or absence of a modulating substance other than cytochrome *b*₅.

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