A BIOCHEMICAL AND IMMUNOCHEMICAL STUDY OF OVINE ADRENAL CYTOCHROME P-450 REDUCTASE

by

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Thesis

presented in partial fulfillment of the

requirements for the degree of

Master of Science

(Biochemistry)

at the

University of Stellenbosch

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March 1992

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DECLARATION

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

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SUMMARY

This study describes:

(a) the isolation of ovine adrenal cytochrome P-450 reductase,

(b) the preparation of antibodies against this enzyme and

(c) comparitive immunochemical studies with ovine liver and bovine adrenal cytochrome P-450 reductases

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OPSOMMING

Hierdie studie beskryf:

(a) die isolering van skaap bynier sitochroom P-450 reduktase,

(b) die opwekking van antiliggame teen hierdie ensiem en

(c) vergelykende immunochemiese studies met skaap lewer- en bees bynier sitochroom P-450 reduktase.

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ACKNOWLEDGEMENTS

I hereby wish to express my sincere gratitude to the following persons and institutions:

Prof. P.Swart for his guidance with the experiments and the preparation of this thesis.

Dr. D.U.Bellstedt for his assistance with the immunological experiments.

Mr. B.Rehder for his technical assistance.

Mr. K.Botha for his help in collecting adrenal glands.

The CSIR for financial support.

My parents for moral support and emosional guidance.

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ABBREVIATIONS

ABTS	2,2'-azino-di-(3-ethyl-benzenethiazoline-sulphonate-6)
ACTH	Adrenocorticotropic hormone
ADX	Adrenodoxin
ADR	Adrenodoxin reductase
2'AMP	Adenosine 2' monophosphate
Bp	Base pair
BSA	Bovine serum albumin
BTH	Buthylated hydroxytoluene
Cyt	Cytochrome
DTT	Dithiotreitol
DHEA	Dehydroepiadrostenedione
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAD	Flavin adenine dinucleotide (oxidized)
$FADH_2$	Flavin adenine dinucleotide (reduced)
FMN	Flavin adenine mononucleotide (oxidized)
$FMNH_2$	Flavin adenine mononucleotide (reduced)
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
$NADPH_2$	Nicotinamide adenine dinucleotide phosphate (reduced)
Mr	Molecular mass
NB	Naked bacteria
PBS	Phosphate buffered saline

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PMSF	Phenylmethanosulfonylfluoride
SCC	Side chain cleavage
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N, N-tetramethylethylenediamine

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CHAPTER 1

INTRODUCTION

The biosynthesis of the glucocorticoids and the mineralocorticoids take place in the adrenal cortex [1]. During the biosynthesis of these very important steroid hormones cholesterol is converted to corticosterone, cortisol, aldosterone as well as the androgens dehydroepiandrostenedione (DHEA) and androstenedione via a number of hydroxylated steroid intermediates. Adrenal steroidogenesis takes place in the mitochondria and endoplasmatic reticulum of adrenocortical cells [1]. These organelles contain a number of specialized steroid hydroxylating enzyme systems, collectively known as the cytochrome P-450-dependent steroid hydroxylases.

Cytochromes P-450 (cyt P-450) are a unique family of hemoproteins which catalyze the incorporation of one atom of molecular oxygen into the substrate and the other atom into water. The electrons required for the reduction of molecular oxygen is obtained via a short electron transport chain from NADPH. Two types of adrenal cytochromes P-450 can be distinguised; mitochondrial cyt P-450's and microsomal cyt P-450's. The mitochondrial cyt P-450's, cytochrome $P\text{-}450_{11\beta}$ (cyt $P\text{-}450_{11\beta}$) and cytochrome $P\text{-}450_{scc}$ (cyt $P\text{-}450_{scc}$) have an electron transport chain consisting of two electron carrying proteins adrenodoxin reductase (ADXR) and adrenodoxin (ADX). In contrast the microsomal enzymes, cytochrome $P\text{-}450_{17\alpha,lyase}$ (cyt $P\text{-}450_{17\alpha,lyase}$) and cytochrome $P\text{-}450_{C21}$ (cyt $P\text{-}450_{C21}$) are located in the endoplasmatic reticulum and has an electron transport system consisting of only one protein, namely cyt P-450 reductase [1]. Cyt P-450 reductase is a membrane bound flavoprotein with a molecular weight of 80 000 [2]. This enzyme is widely distributed in nature and is found in mammalian and non-mammalian organisms [2, 3, 4,

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5]. The enzyme is responsible for the direct transfer of electrons from NADPH to cyt P-450, which serves as a terminal electron acceptor in different metabolic systems such as the metabolism of steroids, fatty acids, insecticides and drugs [6, 7, 8].

Cytochromes P-450 reductase was isolated for the first time in 1950 from pig liver [9]. The enzyme is found in a variety of organs such as the liver, adrenals, kidneys and lungs and was previously isolated and characterized to a great extent [10, 2]. The general properties of these cyt P-450 reductases from different sources are similar even though the properties and functions of the cyt P-450's which they reduce may vary considerably. In adrenal glands for instance the cyt P-450 system is involved in steroid metabolism, while in the liver it is primarily involved in xenobiotic metabolism.

The aim of this study was to purify cyt P-450 reductase, involved in ovine adrenal steroid biosynthesis, and to prepare antibodies against this enzyme for immunochemical studies. As cyt P-450 reductase has not been isolated from ovine tissue before, different isolation procedures were evaluated for this purpose. The purified enzyme was characterized with respect to molecular weight, pI, specific activity and absolute absorption spectra. The purified cyt P-450 reductase was used in an immunization procedure to raise antibodies against the enzyme. These antibodies were used in immunochemical studies of ovine adrenal cyt P-450 reductase and in comparative studies with ovine liver cyt P-450 reductase and bovine adrenal cyt P-450 reductase.

An overview of adrenal steroidogenesis is given in chapter two. In chapter three the discovery of cyt P-450 reductase, distrubution and general features of the protein is discussed. This chapter also describes the proposed

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interaction for cyt P-450 binding to the cyt P-450 reductase. The mechanism of electron transport in microsomes is also discussed. In chapter four the isolation of cyt P-450 reductase from ovine liver and ovine and bovine adrenals is described. The characterization of purified ovine adrenal cyt P-450 reductase is also presented in this chapter and similarities to other purified reductases investigated.

Conventional immunization methods require relatively large amounts of protein which was not available. The use of naked bacteria as an antigen carrier is a relatively new technique which was used previously to raise antibodies against lipopolysaccharides, glycoproteins and proteins [11, 12, 13]. Chapter five deals with the preparation of antibodies against ovine adrenal cyt P-450 reductase. In this chapter cross-reactivities between these antibodies and the ovine liver cyt P-450 and bovine adrenal cyt P-450 reductase as well as the mitochondrial ADXR are investigated. The results obtained during this investigation are discussed in chapter six. In chapter seven the experimental procedures used in this study are discribed in detail.

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CHAPTER 2

THE ROLE OF CYT P-450 REDUCTASE IN ADRENAL STEROIDOGENESIS

2.1. The anatomy of the adrenal gland

The adrenal gland is surrounded by a sturdy capsule of connective tissue. This capsule surrounds the two major regions of the gland: the medulla and the cortex [14].

Underneath the surrounding capsule the three regions of the adrenal cortex are found. The outermost region is called the zona glomerulosa. The cells of this region are arranged in small clusters and arcades. The region is well supplied with capillaries and is responsible for the secretion of mineralocorticoids, which control the sodium balance of the body. The zona fasciculata is continuous with the zona glomerulosa and comprises columns of polyhedral cells aligned at right angles to the surface of the gland. The cells are surrounded by straight capillaries oriented in the same direction as the cells. The cells in the zona fasiculata are larger than the cells of the zona glomerulosa and are normally packed with lipid droplets. Glucocorticoids, cortisol and corticosterone are biosynthesized and secreted in the zona fasiculata. These hormones regulate carbohydrate, fat and protein metabolism. Cortisol also plays a role in the last eight to ten days of gestation. The innermost region of the adrenal cortex encircles the medulla and is called the zona reticularis. The cells are dispersed in an anastomosing network and are

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responsible for the biosynthesis of estrogen and androgen [14].

2.2. Biosynthesis of adrenal steroid hormones

All steroids biosynthesized in the adrenal cortex are derived from a common precursor, cholesterol. Cholestrol is stored as cholesterol esters in lipid droplets in the adrenal glands after transfer from the blood [1].

The intermediates in the conversion of cholesterol to the steroid hormones are subjected to many hydroxylation reactions. These reactions take place in the mitochondria and the endoplasmic reticulum by cyt P-450 catalyzed reactions. The cyt P-450's in the mitochondria and the microsomes differ not only in their protein structure but also in their catalytic function. The electron transfer systems of the mitochondia and the microsomes differ considerably. The mitochondrial system consists of a flavoprotein, an iron sulfur containing protein and cyt P-450 while the microsomal system consists of a flavoprotein and cyt P-450. The cyt P-450 dependent reactions of steroid biosynthesis may be devided into two groups, one group responsible for the cholesterol side chain cleavage reaction and 11β and 18-hydroxylation of deoxycorticosterone in the mitochondria. The other group, which includes the cyt $P\text{-}450_{C21}$ and cyt $P\text{-}450_{17\alpha,lyase}$ reactions, are located in the endoplasmic reticulum of the cells of the adrenal cortex. The function of these cytochromes is hydroxylation of specific metabolites in the steroid metabolic pathway.

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In the mitochondria cyt P-450$_{SCC}$ and cyt P-450$_{11\beta}$ are tightly bound to the matrix side of the inner membrane. These hemoproteins are synthesized as larger precursor molecules on the cytoplasmic ribosomes. Cytochrome P-450$_{C21}$ catalyzes the hydroxylation in the C21 position of progesterone and 17α-hydroxyprogesterone while cyt P-450$_{17\alpha,lyase}$ catalyzes the hydroxylation in the 17α position of progesterone, 17α-progesterone and 17α-pregnenolone [1].

A schematic representation of the conversion of cholesterol to cortisol, aldosterone and androstenedione in the microsomes and the mitochondria of the adrenocortical cells is given in Fig. 2.1 [1]. After the release of cholesterol from cholesterol esters by an esterase, it passes through the membrane to the mitochondria where it is cleaved by cyt P-450$_{SCC}$ at the C20-C22 bond to form pregnenolone. Pregnenolone moves out of the mitochondria into the endoplasmic reticulum where reactions catalyzed by cyt P-450$_{17\alpha,lyase}$ and cyt P-450$_{C21}$ take place. Pregnenolone can be converted by 3β-hydroxy-δ^5-steroid-dehydrogenase-δ^5-isomerase (3βH5D) to progesterone. Cytochrome P-450$_{17\alpha,lyase}$ hydroxylates pregnenolone as well as progesterone to yield the 17α-hydroxylated derivates. The ratio of progesterone to 17α-hydroxypregnenolone, depend on which reaction is fasterthe 3βH5D catalyzed reaction or the cyt P-450$_{17\alpha,lyase}$ catalyzed reaction. Progesterone is also hydroxylated at the C21 position by cyt P-450$_{C21}$ to yield 11 deoxycorticosterone. In some animals a fraction of the 17α-hydroxyprogesterone is converted to andostenedione by cyt P-450$_{17\alpha,lyase}$ while the rest is hydroxylated at the C21 position to form 11-deoxycortisol.

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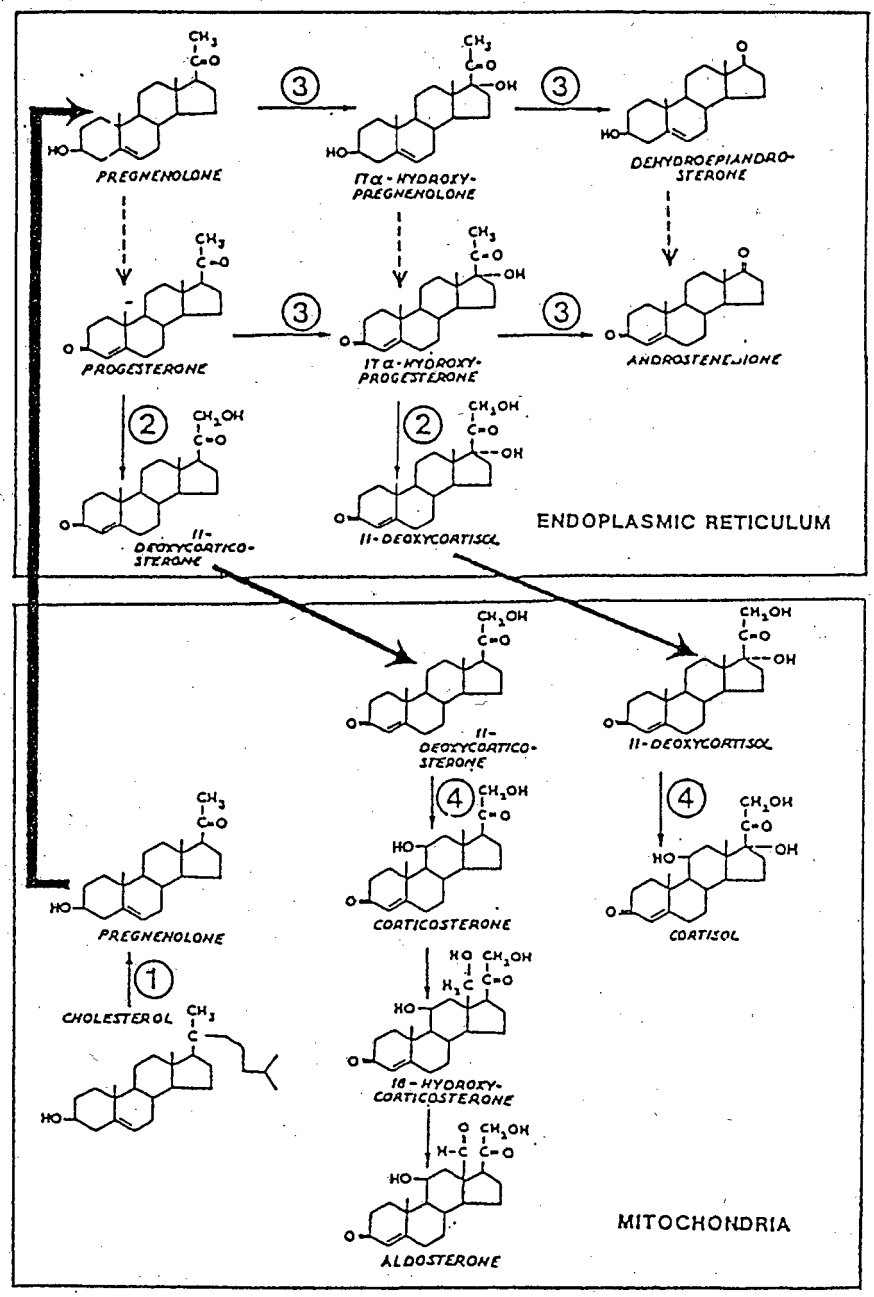

Figure 2.1: Schematic representation of the biosynthesis of adrenal steroid hormones where the solid arrows indicate monooxygenase reaction catalyzed by cyt P-450: (1) $P\text{-}450_{SCC}$ (2) $P\text{-}450_{C21}$ (3) $P\text{-}450_{17\alpha,lyase}$ (4) $P\text{-}450_{11\beta}$. The dotted arrows indicate reactions catalyzed by 3β-hydroxy δ^5-steroid dehydrogenase-δ^5-isomerase. The metabolic intermediates move bask and forth between the two organelle and this is shown by the bold arrows [1].

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Further metabolism of 11-deoxycortisol and 11-deoxycorticosterone occurs in the mitochondria where it is 11β-hydroxylated for the biosynthesis of cortisol and corticosterone respectively. Corticosterone is converted to aldosterone by 18-hydroxylation. These final steroid products corticosterone, cortisol, aldosterone and the androgens, are transported from the mitochondria and microsomes into the blood [15].

2.3. Electron transfer systems involved in adrenal steriodogenisis

Two electron transfer systems are involved in the biosynthesis of steroid hormones in the adrenals, the mitochondrial electron transfer system and the microsomal transfer system. The mitochondrial system is composed of 3 components, ADXR, ADX and cyt P-450. ADXR is a flavoprotein containing one mol of FAD per mol of protein while adrenodoxin is an iron-sulfur protein [16]. Electron transfer procedes as follows:

NADPH → ADXR → ADX → Cyt P-450

A schematic representation of the microsomal and mitochondrial electron transfer systems are shown in Fig. 2.2.

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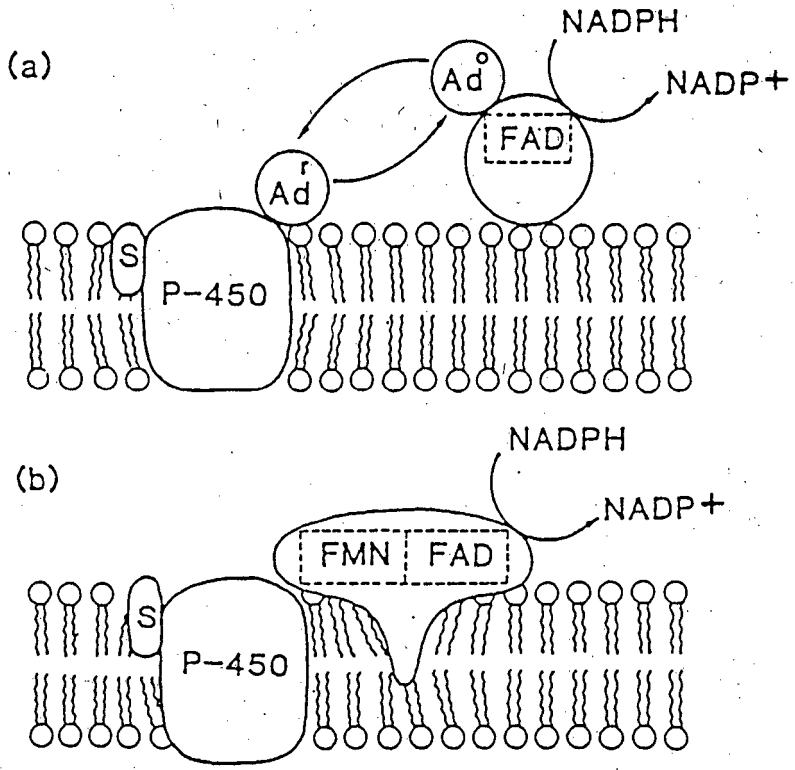

Figure 2.2: Scematic representation of the steriodogenic electron transfer system of the mitochondria (a) and the microsomes (b)[1].

The microsomal electron transfer system comprises a cyt P-450 reductase and cyt P-450. The cyt P-450 reductase is a flavoprotein containing FAD and FMN as prosthetic groups [17]. The native substrate of this enzyme is cyt P-450 but it can also reduce a number of artificial electron acceptors such as cyt b_5 and cyt c. The electron donor of this system is NADPH. The NADPH is oxidized to $NADP^+$ and the cyt P-450 reductase is reduced. The reduced reductase donates the electrons to cyt P-450 which in turn transfer these electrons to the substrate, cyt P-450.

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CHAPTER 3

CYT P-450 REDUCTASE

3.1. Introduction

Horecker [9] first reported a NADPH-mediated cyt c reductase in animal tissue in 1948 and in 1950 he described the purification of this enzyme from whole pig liver using trypsin digestion. The molecular weight (Mr) of the enzyme was determined and it was established that it contained one mol of FAD per one mol of cyt c reductase. Horecker [9] was, however unable to locate the exact cellular or subcellular origin of the cyt c reductase.

In 1955 Strittmatter and Velick [18, 19] published their studies on microsomal cyt b_5 and cyt b_5-reductase. In their experiments they observed a separate microsomal fraction which catalyzed cyt c reduction 20 times faster with NADPH as electrondonor than with NADH. It was subsequently suggested that this enzyme was similar to the enzyme previously isolated by Horecker [18, 19]. In the same year De Duve *et al.* [20] published the results of their studies regarding the intracellular distribution of various enzymes in rat liver. They found NADPH cyt c reductase activity in two fractions, the mitochondria and the microsomes. The NADH cyt c activity was found only in the mitochondria and this enzyme was partly dependent on a antimycin A sensitive factor. The cyt c reductase in the microsomes was entirely insensitive to antimycin A. It was suggested that the distribution patterns observed for the two cyt c reductase activities reflected the existance of two distinct enzyme systems, located in the mitochondria and in the microsomes respectively [20]. Omura *et al.* [21] later confirmed that two different electron transfer systems exist in the

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mitochondria and the microsomes. The non-heme iron protein, ADX, was required in the mitochondria in addition to a flavoprotein for the reduction of cyt c. In the microsomes only a flavoprotein was required for cyt c reduction [21]. In addition it was found that cyt c is confined to the mitochondria while the NADPH cyt c reductase is located in the microsomes [22]. It was then concluded that the microsomal cyt c reductase participates in the microsomal electron transport system that does not involve cyt c as an electron acceptor [22].

Williams and Kamin [23] solubilized a microsomal cyt c reductase by lipase digestion. The cyt c reductase isolated by Horecker [9] and later by Williams and Kamin [23] proved to be identical. The two enzymes had similar prosthetic groups and molecular masses. Both apo-enzymes could be reactivated by both FAD and FMN and the active enzymes had comparible pH optima. In addition, both enzymes were competitively inhibited by NADH [23]. Phillips and Langdon [24] isolated a cyt c reductase from microsomes by solubilization with trypsin and suggested that it was similar to the enzyme previously isolated by Horecker [9].

In 1955 Williams [25] made the first observation of a carbonmonoxide-binding pigment in rat liver microsomes with an intense absorbance in its reduced carbonmonoxide-bound form at 450 nm. Omura and Sato [26] later proved the pigment to be a hemoprotein and concluded that the new cyt is of the b-tipe. Estabrook [27] reported the participation of cyt P-450 as terminal oxygenase in the C21 hydroxylation reaction in adrenocortical microsomes. In 1964 Omura and Sato [28] published their studies on the basic properties of this hemoprotein in its membrane bound (cyt P-450) and solubilized (cyt P-420) forms. In the same year Orrenuis and Ernster [29] reported the selective increase in cyt P-450 and cyt P-450 reductase in liver microsomes of drug-treated

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animals. The following year they reported the parallel increase of cyt P-450 and cyt P-450 reductase by phenobarbital administration to rats. They suggested that the two microsomal components participated in NADPH-mediated drug oxidation [29]. Lu and Coon [30] solubilized, separated and purified the microsomal cyt c reductase, now called cyt P-450 reductase, by treatment of microsomes with detergent and for the first time the cyt P-450 mediated monoxygenase system could be reconstituted *in vitro*.

In 1971 Masters *et al*. [31] showed, with immunochemical studies, that the cyt P-450 reductase found in liver microsomes and the enzyme found in the adrenal cortex were similar. They also showed that the cyt P-450 reductase in the adrenal cortex and liver microsomes were immunochemically distinct from the protein responsible for cyt P-450 reduction found in the adrenocortical mitochondria [31]. It is now generally accepted that the adrenocortical and the hepatic microsomal cyt P-450 reductase are similar as the protein has been characterized to a great extent with respect to prosthetic groups, different binding areas and Mr [32, 33, 34].

The enzyme is identical to the cyt P-450 reductase found in other tissues such as the liver, lung and kidney [10]. The flavoprotein has been isolated from various types of tissue from a variety of animals [2, 3, 35, 36, 37, 38], yeasts [4, 39] and plants species [5]. The microsomal cyt P-450 reductase is responsible for the direct transfer of electrons from the NADPH to cyt P-450 in the endoplasmic reticulum. Cytochrome P-450 serves as a terminal electron acceptor for the metabolism of steroids, fatty acids, insecticides, alkanes, drugs and carcinogens in a variety of tissues [6, 7, 8]

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Cytochrome P-450 reductases from different organs in the same animal are thought to be coded by a single gene. This idea is supported by the fact that adrenal microsomal cyt P-450 reductase have properties similar to the hepatic microsomal enzyme with regard to prosthetic groups, pH optima, dependence of enzyme activity on ionic strength of the solution and NADPH specificity [2, 31, 40].

3.2. Structural features of cyt P-450 reductase

The cyt P-450 reductase is an amphipatic molecule, consisting of a hydrophobic domain (responsible for binding the cyt P-450 to the membrane) and a hydrophilic catylitic domain [41, 36]. The rat liver cyt P450 reductase contains α-helix, β-sheet and random structures in the following percentages 33,8%, 16,4% and 1,9% respectively [42]. The cyt P-450 reductase is not bound to any carbohydrate and contains no metals [2, 43]. It consists of 5 structural domains, two substrate binding segments (NADPH and cyt P-450), two flavin binding segments (FAD and FMN) and a membrane binding domain [32].

3.2.1. Structure of the NADPH-binding domain

The secondary structure of the NADPH binding site of ovine liver cyt P-450 reductase was reported to be predominantly β-sheet [17]. A cysteinyl residue has a functional role in the NADPH binding domain of the enzyme from several species [44, 2]. The cysteinyl containing peptides of the porcine liver enzyme shows a high degree of sequence homology to other flavoproteins such as glutathione reductase, D-amino acid oxidase, p-hydroxybenzoate hydroxylase and flavodoxin. The functions of the cysteinyl residues are not the same in all these enzymes [17]. These residues are protected from S-

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alkylation in the presence of NADPH which strongly suggests its location to be very close to, or in the NADPH binding site [17, 2]. An arginyl residue and a histidyl residue are reported to be essential for the enzymatic activity of the cyt P-450 reductase [45, 2]. These positively charged groups are able to form stabilizing salt bridges with the acidic pyrophosphate group of the NADPH.

The catalytic activities of cyt P-450 reductase towards different substrates are sensitive to changes in the ionic strength of the meduim [23, 2]. This phenomenon could be related to the interference with the ionic binding of the 2'monophosphate ester group of the NADPH to the NADPH binding site [24, 2]. The activity towards different substrates increased with an increase in ionic strength. The metabolism of the different substrates were however not influenced to the same extent [2]. $NADP^+$ inhibits the oxidation of NADPH by competing for the same bindingsite [2].

3.2.2. Structure of the cyt P-450 binding domain

The cyt c and cyt P-450 binding domains are thought to be located in close proximity to each other [32]. The detergent solubilized cyt P-450 reductase is active in reducing cyt P-450 [30, 46] and cyt b_5 [47] whereas the protease solubilized enzyme is not functional in reducing either [34, 47, 48, 41]. Protease solubilized and detergent solubilized cyt P-450 reductase can however reduce cyt c [34]. Chemical modification studies with rat liver cyt P-450 reductase showed that acidic amino acid residues on the enzyme are involved in the interaction and electron transfer between the cyt P-450 reductase and the cyt P-450 [49]. The carboxyl groups on the cyt P-450 reductase interacts with the positively charged cyt P-450. The modification of some of

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these carboxyl groups of the cyt P-450 reductase caused a dramatic decrease in the ability of the enzyme to interact with the cyt P-450. These findings suggest that the two proteins are in a specific orientation relative to each other as illustrated in Fig. 3.1 [49].

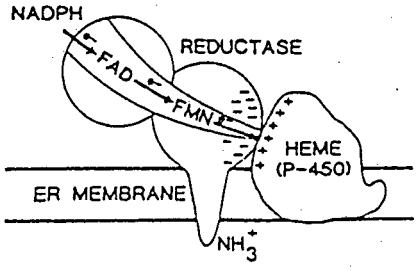

Figure 3.1: Schematic representation of the interaction of cyt P-450 reductase with cyt P-450 to ensure optimal electron transfer.

This orientation is necessary for maximal interaction and electron transfer between the cyt P-450 and the cyt P-450 reductase. It is evident that electrostatic interactions plays an important role in these charge-pair interactions [50, 51].

Gut *et al.* [52] suggested the possibility of a mixed type electron transfer mechanism containing both stable binary complexes between cyt P-450 and cyt P-450 reductase and lateral collision controlled electron transport between independently diffusing components. In rat liver microsomes only 5% of the cyt P-450's are associated in complexes with the cyt P-450 reductase [52]. Hydrophobic interactions between the cyt P-450 and the cyt P-450 reductase are however not ruled out, because both these proteins contain hydrophobic regions. Compelling evidence for the free association of the cyt P-450 reductase and cyt P-450 in liposomal membranes was provided by Taniguchi *et al.*[53]. A decreasing phospholipid content results in an increase in the cyt P-450 reduction rate [52]. The reaction can be

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decribed by a simple mass action model proposed by Miwa *et al*. [54].

Cyt P-450 + Cyt P-450 Reductase ⇌ Cyt P-450-Cyt P-450 reductase

The dissociation constant for the formation of this transient complex in liposomes was found to be 0,1 μM [55]. Results suggest that the dissociation constant for this complex is independent of the membrane or monomeric state of the lipid [55].

3.2.3. Structure of the FMN and FAD binding domains

Nisimoto and Shibata [44] demonstrated that the FMN may associate with the cyt P-450 reductase by FMN-Trp interactions. Chemical modification of a couple of Trp residues can account for the inability of FMN to rebind to FMN-depleted cyt P-450 reductase [44]. Cloning of the rat liver cyt P-450 reductase in *E. coli* yieled an enzyme with similar properties as the native enzyme. Replacement of Tyr 178 with Asp caused a loss in FMN binding ability and cyt c reductase activity. A substitution of Tyr 140 with Asp resulted in a 5 fold reduction in cyt c reductase activity, but had no effect on FMN binding. This suggests that the FMN bound in a conformation which does not permit efficient electron transfer. A total loss of FMN binding occured when Tyr 178 alone or Tyr 178 and Tyr 140 were substituted with Asp in mutants. A reduced incorporation of FAD into the newly synthesized mutant protein occured which suggests that the FMN binding may help with FAD incorporation [33].

Porcine liver cyt P-450 reductase contains a flavin binding domain near the C-terminus thought to be the FAD binding domain [17]. This fragment contains a sequence Gly-X-Gly-Y-Gly which is an invariant sequence for flavin binding domains which is homologous to the flavin binding areas of

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flavodoxin, lactate hydrogenase, p-hydroxybenzoate hydroxylase and D-amino acid oxidase.

The FAD and FMN moieties are non covalently associated with the protein since these groups were completely dissociated under acidic conditions [17]. Crossover binding of the FMN to the FAD binding site occured under certain conditions in FAD depleted cyt P-450 reductase. Spectral evidence indicates that FMN remains in its native state even after FAD removal [56]. As FMN and FAD have different oxidation-reduction potentials, it is evident that they will have distinct roles during catalysis [57].

3.2.4. Structure of the membrane binding domain

The membrane binding segment is rich in hydrophobic residues like Gln and Asn [34] and serves as an anchor for the flavin containing portion at the surface of the membrane [58]. The membrane binding segment of rabbit liver cyt P-450 reductase is suggested to be a 17-residue segment from Val 16 to Phe 32 [41]. The residues in this segment are mostly Val, Ile, Tyr and Trp which show a strong tendency for β-sheet formation. This would permit the polipeptide segment to span the lipid bilayer. On either side of the hydrophobic region a considerable number of charged residues are found [36]. The hydrophobic segment may participate in binding the cyt P-450 reductase to the membrane or in orientating the enzyme for maximal interaction with cyt P-450 [34]. A catalytic role for this segment can, however, not be ruled out [59, 60].

The mode in which the hydrophobic domain is bound has not been documented, but two conformations is seen as alternatives for the orientation of the liver and the adrenal cyt P-450 reductase membrane binding domain [36,

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40]. One model suggests the N- and C-terminal on opposite sides of the membrane with the hydrophobic segment spanning the membrane. A second model suggests both termini on the same side of the membrane with the apolar region embedded in a "U" or hairpin fashion as has been proposed for cyt b_5 (Fig. 3.2) [36, 61].

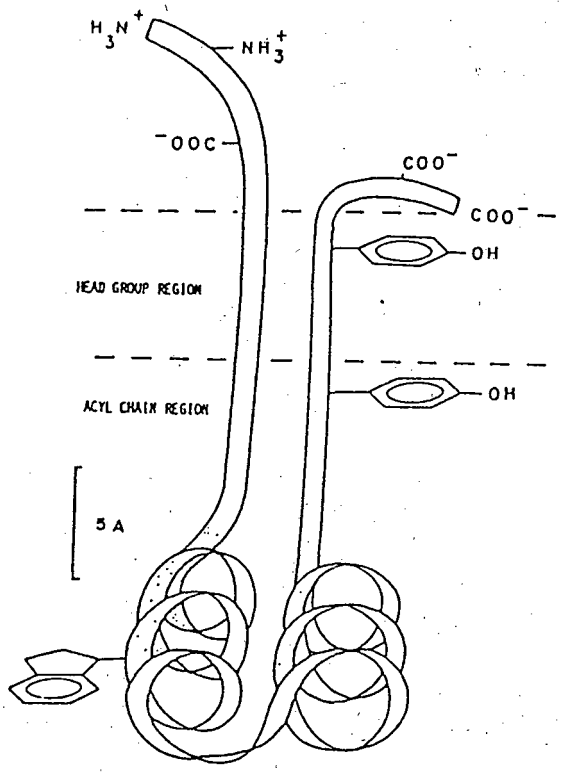

Figure 3.2: Structural orientation of the membrane binding segment of cyt b_5.

The carboxy and amino termini of the human liver cyt P-450 reductase is accessible to each other during the catalytic action of the enzyme which could indicate a hairpin fashion

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arrangement [62]. Cytochrome P-450 reductase can only reduce cyt b_5 if both proteins are detergent solubilized, which indicates the requirement for the membrane binding segment for efficient catalytic interaction [47]. The hydrophobic component is necessary for demethylation and hydroxylation reactions [63].

3.2.5. Structure of the C-terminus and N-terminus

It was reported that the C-terminus of porcine liver cyt P-450 reductase contains a hydrophobic domain. In the last 48 residues 6 basic and no acidic residues are found. The rest of the residues are neutral or hydrophobic. The segment from residue number 21-48 contained the hydrophobic domain with 9 aromatic residues. It is suggested that at neutral pH the C-terminus may be charged [17]. The C-terminal sequence of rat liver cyt P-450 reductase is Trp-(Leu, Val)-Asp-Ser-COOH [41].

In the rabbit liver cyt P-450 reductase the N-terminal segment from Gly-1 to Ser-9 contains only hydrophobic and ionic residues. From Arg-33 to Lys-44 two anionic and 5 cationic residues are found clustered at the beginning of this segment. This segment is found at the endoplasmic reticulum-cytosol interface [36]. The N-terminal sequence for porcine, rabbit and rat liver is highly conserved as can be seen from the amino acid sequences in Fig. 3.3 [17].

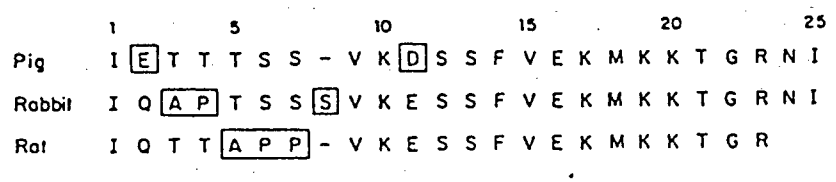

Figure 3.3: N-terminal sequences of trypsin solubilized porcine, rabbit and rat liver cyt P-450 reductase. Substituted amino acids are boxed in.

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3.3. Models proposed for the association of cyt P-450 and cyt P-450 reductase with the microsomal membrane.

There are two proposed models for the association of cyt P-450 and cyt P-450 reductase in the membrane, a "rigid" and a "non-rigid" organization. The "rigid" model suggests that there are several molecules of cyt P-450 associated rigidly around each cyt P-450 reductase [64]. The "non-rigid" model proposes that the cyt P-450 reductase can associate with all cyt P-450 molecules and with cyt b_5 which can all be reduced enzymatically [65]. In a "non rigid" system inter-complex electron transfer is possible, but not in a "rigid" system [64, 65]. Yang [65] supported the "non-rigid" model, while French *et al*. [35] favoured the idea of complex formation.

Similar studies on the cyt b_5 system lent support to random distribution and rapid lateral mobility of the cyt b_5 system in the membrane [66]. Cytochrome P-450 and cyt P-450 reductase however do not have translational movement as fast as have been reported for cyt b_5 and the cyt b_5 reductase [66, 67]. A single cyt P-450 reductase molecule can reduce several molecules of cyt P-450 through lateral diffusion [54]. If the cyt P-450 and cyt P-450 reductase were diluted with lipid in a reconstituted system the activity of a "rigid" association should not be affected. It was however found that the reduction of cyt P-450 was limited. This effect could not be explained by the cluster or "rigid" model [53]. The stoicheometry of the catalytic active cyt P-450 and cyt P-450 reductase has been shown to be 1:1 in a non-membranous reconstituted system [35]. Maximum activity was observed in a liposome reconstituted system when the concentration of the two proteins reached equivalency.

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3.4. The role of the membrane binding segment in the biological function of the cyt P-450 reductase

3.4.1. The role of the cyt P-450 reductase membrane binding segment in microsomal membranes

Detergent solubilized, affinity purified cyt P-450 reductase interacts with microsomes illustrating the hydrophobic nature of cyt P-450 reductase. Detergent solubilized cyt P-450 reductase is fully functional in reducing cyt c and cyt P-450. Upon protease solubilization of the enzyme however the membrane binding segment remains in the membrane. The segment remaining in the membrane does not prevent the binding of additional cyt P-450 reductase molecules to the membrane [34].

The binding of detergent solubilized cyt P-450 reductase to phospholipid vesicles is spontaneous and rapid. This indicates that no other membrane components are required for binding. There appears to be some interaction between the cyt P-450 reductase and hydrophobic portions of the lipid bilayer as high salt concentrations does not disrupt this attachment [34].

3.4.2. The role of the cyt P-450 reductase membrane binding segment in binding cyt P-450

Protease solubilized cyt P-450 reductase can reduce artificial electron acceptors but not its native substrate cyt P-450 [46, 68]. This implies that the membrane binding segment in some way facilitates catalytic interaction between the cyt P-450 and the cyt P-450 reductase [69, 36, 47]. There are two possible ways in which the hydrophobic portion of the cyt P-450 reductase could function; the cyt P-450 reductase could act as a hydrophobic anchor for the

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cyt P-450 (Fig. 3.4A) or the two proteins could interact through specific interaction (Fig. 3.4B) [69].

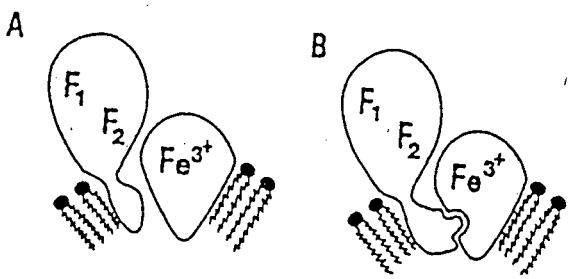

Figure 3.4: Cytochrome P-450 reductase may act as a hydrophobic anchor for the cyt P-450 (A) or through specific protein-protein interactions (B).

If specific protein-protein interactions occur during the binding of the cyt P-450 reductase to the cyt P-450, purified membrane binding segments would compete with the functional cyt P-450 reductase for cyt P-450 binding, which would result in an inhibition of hydroxylation [69]. The hydrophobic segment of the cyt P-450 reductase isolated by Black *et al.* [41] inhibited the monoxygenase reaction in reconstituted sytems, while Gum and Strobel [69] found just the opposite. The reason for these totally different results are unclear.

Similar interaction as proposed for the cyt P-450 and cyt P-450 reductase, might exist between cyt P-450 and cyt b_5. The simplest mechanism of interaction of the three membrane proteins would be a reversible association. This association would result in the formation of dimers [51]. Electron transfer from the cyt P-450 reductase to cyt P-450 or cyt b_5

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or from cyt b_5 to cyt P-450 would then occur during the existance of these dimers.

3.5. The effect of cyt P-450 reductase cleavage on its biological function

3.5.1. Properties of uncleaved detergent solubilized cyt P-450 reductase

Based on its behaviour in solutions, in the presence or absence of detergents, phospholipid vesicles and microsomal membranes it may be deduced that detergent solubilized cyt P-450 reductase is an amphipathic molecule [34]. Protease-solubilized cyt P-450 reductase is unable to reduce its native electron acceptor cyt P-450, although it reduces artificial electron acceptors such as cyt c [47, 48, 41]. The detergent solubilized form however retains the ability to reduce both types of electron acceptors [70, 34] including cyt b_5 [47, 71, 41, 2]. This catalytic difference is paralleled by a difference in subunit molecular weight. The protease solubilized form has a Mr of 8 500 to 13 000 Da less than the detergent solubilized form [34, 72, 23, 20] depending on which protease is used for solubilization. The membrane binding segment which is removed from the catalytic portion of the cyt P-450 reductase upon protease solubilization is essential for the enzyme's biological function [36, 47], which is the reduction of cyt P-450.

3.5.2. Properties of trypsin cleavaged cyt P-450 reductase

It was indicated by Black *et al.* [41] that tryptic cleavage of cyt P-450 reductase separated the protein into two functional domains. The larger fragment represents the

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catalytic domain and is more hydrophilic than the native enzyme [36]. The smaller fragment is hydrophobic and plays a role in binding the native enzyme to the microsomal membrane [34, 41]. In porcine liver cyt P-450 reductase the hydrophobic portion accounts for 5 kDa of the protein [17].

It was reported that detergent solubilized cyt P-450 reductase and trypsin solubilized cyt P-450 reductase have the same C-terminus [30]. This indicates that the membrane binding portion is located on the N-terminus of the native enzyme. The membrane binding portion of the cyt P-450 reductase has a uniquely sensitive site for trypsin cleavage between Lys 56 and Ile 57. This bond demarcates the membrane binding domain from the catalytic portion of the enzyme [30]. The treatment of cyt P-450 reductase with trypsin led to a loss in cyt P-450 reduction activity, but the ability to reduce cyt c was retained [58]. The hydrophobic protein portion removed by trypsin treatment is 10% of the total protein contents of the enzyme [58]. Trypsin cleaved cyt P-450 reductase contains one mole of FMN and one mole of FAD indicating that the trypsin cleavage has no effect on the binding of these flavins and that they are located in the catalytic portion of the cyt P-450 reductase [63].

3.5.3. Properties of cyt P-450 reductase treated with steapsin

Steapsin solubilized rat liver cyt P-450 reductase has a membrane binding domain which is not esspecially apolar. This could indicate that only a portion of the membrane binding segment is embedded in the membrane, or that a small fragment of the hydrophilic portion of the cyt P-450 reductase is removed upon solubilization [69]. This cyt P-450 reductase has a Mr of 78 000. When cyt P-450 reductase was incorporated in deoxycholate micelles and treated with steapsin a transient 74 500 Da species was formed [34]. This

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species gradually disapeared and a 68 000 Da species was formed which is thought to be cyt c reductase [34]. This conversion of cyt P-450 reductase to cyt c reductase correlates with a decrease in NADPH-dependent benzphetamine demethylation but had no effect on the cyt c reductase activity. This decrease could be related to the inability of the cyt c reductase to interact with the substrate, benzphetamine [34].

3.5.4. The effect of alkaline phosphatase treatment on the properties of cyt P-450 reductase

Phosphatases remove phosphate groups from proteins. Treatment of microsomes with alkaline phosphatase resulted in a time dependent decrease in the monooxygenase activity. One or both of the main components (Cyt P-450 and Cyt P-450 reductase) of the monooxygenase system were effected by dephosphorylation. A decrease of cyt P-450 reductase activity was found, which was in the same order of magnitude as that of the decrease in monooxygenase activity [73, 74]. It was established that cyt P-450 was unaffected by the phophatase treatment and that the decrease in the monoxygenase activity was solely the effect of a dephosphorylation of the cyt P-450 reductase. The decrease in isolated cyt P-450 reductase activity followed a time course similar to that of the microsome bound cyt P-450 reductase [74]. These findings suggest that the native cyt P-450 reductase is a phosphorylated protein and dephosphorylation causes an activity change in this enzyme [73].

The mechanism of action of the phosphatase on the cyt P-450 reductase is not clear. Indications are that the prostetic group of the cyt P-450 reductase are attacked [73]. In the phosphatase treated enzyme a flavin analysis showed the

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appearance of riboflavin with a concomitant decrease in FMN [74]. About 80% of the FMN is converted to riboflavin, but the FAD was unaffected because the phosphatase lacks pyrophosphatase activity [74]. Ferricyanide reduction was not affected by phosphatase treatment but a decrease in cyt c reductase activity was found [74]. FMN-depleted cyt P-450 reductase is unable to reduce cyt c and cyt P-450 but retains the ability to reduce ferricyanide [75, 76]. These facts support the hypothesis that the phosphatase attacks the cyt P-450 reductase by reacting with the FMN in the enzyme [74].

3.6. The mechanism of electron transport in liver microsomes

The electron transport in liver microsomes procedes from NADPH to FAD, FMN and cyt P-450 [77, 78, 79, 80]. The electron transport mechanism has been studied extensively [81, 82]. The catalytic cycle consists of alternations of flavin between the fully reduced and the half reduced forms. The fully oxidized flavin does not appear to participate in catalysis [43].

The air stable semiquinone form was reported to be a one-electron-reduced species, which could be either FADH$^{\bullet}$-FMN or FAD-FMNH$^{\bullet}$. The semiquinone derived from FMN depleted enzymes was unstable in O_2. Thus the air stable semiqiunone was identified as being FAD-FMNH$^{\bullet}$. When treated with NADPH a decrease in the cyt P-450 reductase absorbance at 455 nm was accompanied by a negligible change of absorbance at 585 nm. The oxidized FAD accepts two electrons from NADPH. One is transfered intramolecularly to FMNH$^{\bullet}$. The concentration of each redox state is governed by the NADPH concentration. At higher molar ratios of NADPH to cyt P-450 reductase the three-electron-reduced species FADH$^{\bullet}$-$FMNH_2$ is increased. The

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air stable semiquinone is inactive towards several electron acceptors [76] and it is rapidly reduced by NADPH to the three-electron-reduced state which can donate two electrons to cyt P-450. The proposed mechanism for this electron transfer is summerized in reactions 1-5 [76].

$$\text{FAD-FMN} + \text{NADPH} + H^+ \rightleftharpoons \text{NADP}^+\text{-FADH}_2\text{-FMN} \quad (1)$$

$$\text{NADP}^+\text{-FADH}_2\text{-FMN} \rightleftharpoons \text{NADP}^+\text{-FADH}\cdot\text{-FMNH}\cdot \rightleftharpoons \text{FAD-FMNH}_2 + \text{NADP}^+ \quad (2)$$

$$\text{FAD-FMNH}_2 + \text{RH-Fe}^{3+}\text{ (P-450)} \rightleftharpoons \text{FAD-FMNH}\cdot + \text{RH-Fe}^{2+}\text{ (P-450)} + H^+ \quad (3)$$

$$\text{FAD-FMNH}\cdot + \text{NADPH} + H^+ \rightleftharpoons \text{NADP}^+\text{-FADH}_2\text{-FMNH}\cdot \quad (4)$$

$$\text{NADP}^+\text{-FADH}_2\text{-FMNH}\cdot = \text{NADP}^+\text{-FADH}\cdot\text{-FMNH}_2 + \text{RH-Fe}^{2+}O_2\text{ (P-450)} \rightleftharpoons \text{NADP}^+\text{-FADH}\cdot\text{-FMNH}\cdot = \text{FAD-FMNH}_2 + \text{NADP}^+ + \text{Fe}^{3+}\text{ (P-450)} + \text{ROH} + OH^- \quad (5)$$

The FAD-FMNH form is the air stable semiquinone and the RH-Fe^{3+}(P-450) is the substrate bound oxidized form of the cyt P-450 [76]. The three-electron-reduced form can supply the first and the second electrons to the cyt P-450 (reaction 6 and 7). FAD is able to accept two reducing equivalents whereas FMN appears to serve as a one electron carrier in the process of electron transfer from NADPH to cyt P-450 during the mixed function oxidase catalytic cycle [76].

$$\text{NADP}^+\text{-FADH}_2\text{-FMNH}\cdot = \text{NADP}^+\text{-FADH}\cdot\text{-FMNH}_2 + \text{RH-Fe}^{3+}\text{ (P-450)} \rightleftharpoons \text{NADP}^+\text{-FADH}\cdot\text{-FMNH}\cdot = \text{FAD-FMNH}_2 + \text{NADP}^+ + \text{RH-Fe}^{2+}\text{ (P-450)} + H^+ \quad (6)$$

$$\text{FAD-FMNH}_2 + \text{RH-Fe}^{2+}O_2\text{ (P-450)} \rightleftharpoons \text{FAD-FMNH}\cdot + \text{Fe}^{3+}\text{ (P-450)} + \text{ROH} + OH^- \quad (7)$$

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3.7. The effects of adrenocorticotropin (ACTH) on the cyt P-450 reductase activity

ACTH regulates the adrenal steroidogenic pathway and adrenal cells treated with this hormone showed an increase in the concentration of cyt P-450 [83, 84]. Dee *et al*. [10] showed that the concentrations of cyt P-450 reductase in bovine adrenal cells are increased upon ACTH treatment. Within 24 hours the rate of cyt P-450 reductase synthesis of the treated cells were twice that of the untreated controls. The increase in cyt P-450 reductase synthesis optimized at approximately the same time as that of cyt P-450 [83, 85]. The newly synthesized cyt P-450 reductase had the same Mr as the mature purified enzyme. This agrees with reports indicating that the rat liver cyt P-450 reductase is synthesized in its mature form [10, 86] and not as a larger precursor molecule. The synthesis of the protein maximizes 24 hours after initiation of the treatment and the activity maximizes 12 hours later. This time difference could be related to binding of the prosthetic groups FMN and FAD to the inactive cyt P-450 reductase molecule [10].

ACTH regulates the cyt P-450 reductase in the adrenocortical cells via cAMP. Reduction is however also induced by ACTH and cAMP analogs but not by phenobarbital [10]. Phenobarbital induction of cyt P-450 reductase is limited to the liver. The expression of this gene is regulated by different factors in different tissues indicating a very specific regulation of expression [10].

3.8. Characterizing of the cyt P-450 reductase gene

The rat liver cyt P-450 reductase has been characterized by Gonzalez and Kasper [87] and Porter *et al*. [88]. The total exon length is about 2 600 bp (base pairs) which corresponds favourably with the size of the cyt P-450 reductase mRNA

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[87]. Porter *et al.* [88] confirmed that a single form of cyt P-450 reductase exists in the rat liver. The gene structure of this enzyme is represented in Fig. 3.5. On the gene, basis +1 to +238 represents a non-coding region as no AUG codon is present [87].

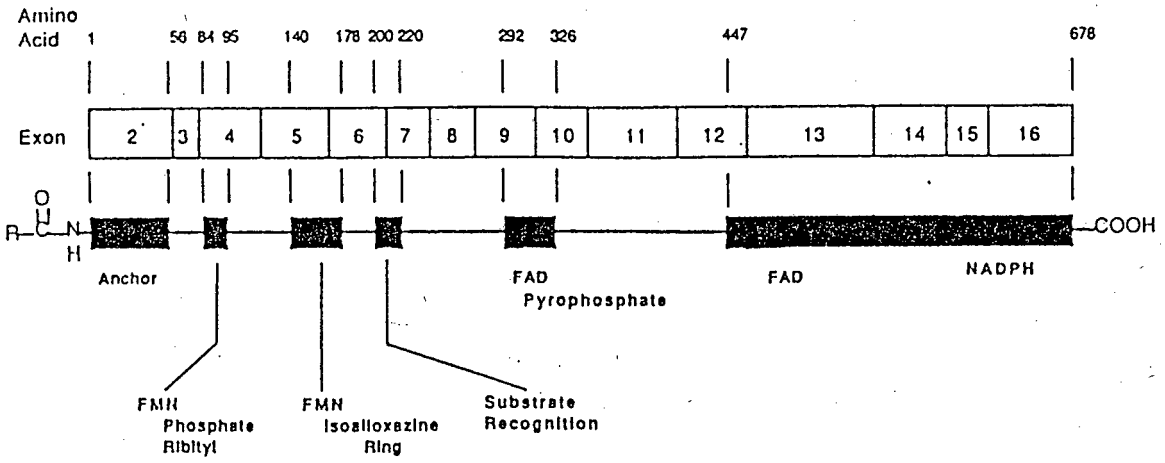

Figure 3.5: The gene and protein structure of rat liver cyt P-450 reductase

On the rat gene exon 2 represents the hydrophobic membrane binding segment and the small exon 3 encodes the segment which serves as a bridge between this segment and the FMN binding domain. Exons 4, 5 and 6 encodes the FMN binding domain where exon 4 encodes the phosphate binding residues and exons 5 and 6 encodes the essential tyrosine residues (Tyr 140 and 178) involved in the binding of FMN. Exons 6 and 7 encode the acidic residues between 200 and 220 which may be involved in the interaction between the cyt P-450 reductase and cyt P-450 [32, 88]. The FMN and FAD domains are joined by a peptide encoded by exons 7 and 8. Exon 9 encodes the segment interacting with the pyrophosphoryl portions of the FAD molecule. Exons 10-12 encode a peptide which may be involved in the orientation of the FMN and FAD domains so as to facilitate electron transfer between the two flavins. Part of exon 13 encodes the beginning of the

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NADPH binding domain including the pyrophosphate binding residues. The rest of this domain is encoded by the exons 14-16 [88].

The homology of the N-terminal portion of the rat cyt P-450 reductase with flavodoxin (containing FMN as prosthetic group) and the C-terminal homology with ferredoxin $NADP^+$ reductase (a FAD containing protein) suggest that the P-450 reductase arose through a fusion of the ancesteral genes for these two functionally linked flavoproteins [89]. It seems reasonable to hypothesize that the genes for flavodoxin and ferredoxin $NADP^+$ reductase were arranged in tandem in an operon of an organism. At some point it fused to give a P-450 reductase precursor. In the present enzyme approximately 40 amino acids separate the FMN and the FAD binding domains as seen in Fig. 3.6. FMN binding domain represents the region in the cyt P-450 reductase homologous with ferrodoxin $NADP^+$ reductase. FNR represents a portion of the cyt P-450 reductase homologous to residues 20-55 of the ferredoxin-$NADP^+$ reductase molecule [89].

The origin of this short segment is unclear but it may have been present in a precursor protein or evolved to enhance the coupling of the two flavin domains. This stretch of residues are almost entirely α-helix with the predominance of negatively charged residues. The question that arises now is how the electron transfer is effected between the two domains having a negatively charged bridge between them [89].

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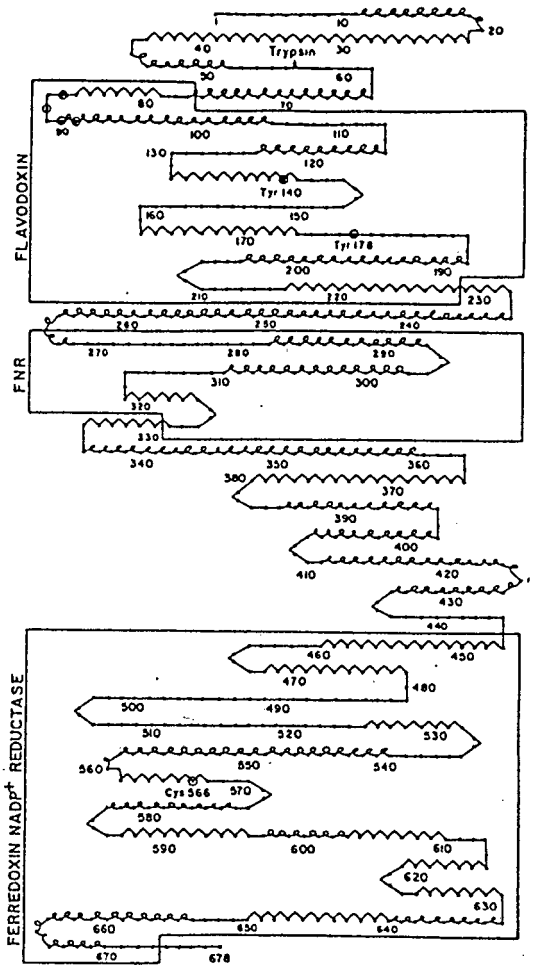

Figure 3.6: Predicted secondary structure of cyt P-450 reductase. Loops indicate α helix; zig zag, β-strand and straight lines, random coils.

3.9. The effects of lipids and cyt b_5 on the cyt P-450 reductase activity

The cyt P-450 reductase does not exhibit a lipid requirement regarding artificial electron acceptors [23, 46] but cyt P-450 require lipids for enzymatic reduction. It was found that the enzymatic activity decreased with a decrease in lipid concentrations [90]. The enzymatic reaction is biphasic, with a rapid and a slow phase. The rapid phase is dependent on the presence of the lipid phase. In the absence of lipid only the slow phase is detected [90].

Microsomal drug metabolism may involve cyt b_5 and NADH-cyt b_5 reductase under certain conditions. It was however shown that reduction of cyt P-450 in the liver microsomes occur in

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the absence of cyt b_5 [48, 91]. Studies on NADPH-mediated reactions in hepatic preparations led to proposals that cyt b_5 plays a fascilitory role in the transfer of the second electron in substrate oxidations [91].

The addition of cyt b_5 to a NADPH-dependent micellar system has led to varied effects on substrate metabolism [92]. Inhibitory and stimulatory effects of cyt b_5 in reconstituted micelles containing the cyt P-450 hydroxylation systems were reported by different laboratories [93, 94, 92]. The effect of cyt b_5 on the cyt P-450 reduction system was tested in artificial membranes such as micelles and vesicles. The effects of cyt b_5 on the cyt P-450 reduction depended greatly on the lipid to cyt b_5 to cyt P-450 to cyt P-450 reductase ratio [92].

Disruption of vesicular membranes with detergent caused a slight activation in the activity of the cyt P-450 reductase. This suggested that only a small fraction of the cyt P-450 reductase was not accesable to either the NADPH or cyt c or both. In reconstituted systems, which lacked NADH and NADH cyt b_5 reductase activity, cyt b_5 reduction is dependend only on its interaction with cyt P-450 reductase, cyt P-450 or both proteins [92]. Increases in reaction rate could involve a direct participation of cyt b_5 in electron transfer or alternatively, allosteric interactions with the two other protein components to facilitate electron flow between them. Evidence for a direct role in electron transfer includes reduction of cyt b_5 by cyt P-450 reductase [47, 63]. In rat liver, immunochemical studies supported the theory that cyt b_5 directly interacts with the cyt P-450 electron transfer system (see Fig. 3.7).

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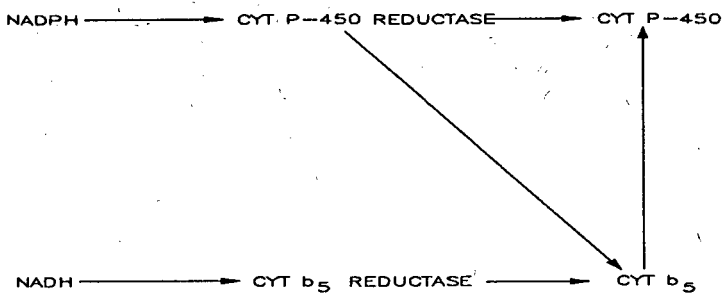

Figure 3.7: Interaction of the cyt P-450 electron tranfer system with the cyt b_5 electron transfer system in rat liver microsomes [95].

It was, however, suggested that cyt b_5 is not an obligatory component of these hydroxylations, but does play a role in regulating the hydroxylation pathways involving the cyt P-450 hydroxylation system [94]. The dependence of activity on the molar ratio of components of the endoplasmic reticulum also illustrates the potential for modulation of metabolic activity by changes in the lateral organization within the membrane [92].

3.10. The presence of cyt P-450 reductase in non mammalian organisms

3.10.1. The presence of cyt P-450 reductase activity in yeast

Growth of *Candida tropicalis* on hydrocarbons induces a cyt P-450 system. This system hydroxylates a variety of fatty acids, hydrocarbons and druggs [4, 39]. A growth source of tetradecane induces the cyt P-450 more than a growth source of glucose. An increase in the hydroxylation components as well as hydroxylation activity is found in *Candida tropicalis* [39]. Spectral evidence for the conversion of the low spin to the high spin states of yeast cyt P-450 was similar to mammalian microsomal cyt P-450 [4, 39]. *Saccharomyces cerevisiae* cyt P-450 reductase has a Mr of

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Saccharomyces cerevisiae cyt P-450 reductase has a Mr of 78 000 and absorbtion maxima at 385 and 455 nm. Both these features compare well with the results found for the liver and adrenal cyt P-450 reductases [2, 42]. It was reported earlier [91] that mixed function oxidation reactions in liver microsomes are stimulated to different degrees by the addition of NADPH and NADH. The yeast cyt P-450 reductase has the same pyridine specificity as the microsomal enzyme and the system is more active with NADPH than NADH as a electron donor [39, 4]. A combination of both cofactors had a synergistic effect on the biological activity of the enzyme.

3.10.2. The presence of cyt P-450 reductase activity in plants

Cytochrome P-450 reductase was isolated from *Cathatanthus roseus* and had Mr's of 78 000 and 63 000. Like the mammalian enzyme the activity was dependent on ionic strength [3, 81]. Isoelectric focussing showed the pI of this enzyme to be 5,3 which is in close accordance with values of 5,1 and 5,2 previously reported for the rabbit liver cyt P-450 reductase [96, 5]. In the plant *Helianthus tuberosus* the cyt P-450 reductase activity was very low. When the plant was wounded, however, the monooxygenase system was induced. The induced cyt P-450 reductases had Mr's of 80 000, 82 000 and 84 000 which are higher than that found for the mammalian enzyme [2, 71]. Immunological studies showed no cross reactivity between the plant cyt P-450 reductase and the mammalian cyt P-450 reductase antibodies and vice versa. The three forms of cyt P-450 reductase found in the plant is not so unusual since Dee *et al.* [10] discovered multiple forms of cyt P-450 reductase in animal systems. A steep increase 2 hours after wounding occured pointing to the activation of an inactive pro-enzyme. It is however not clear whether this effect of

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sudden increase is due to enhanced transcription, post translational modification or pre-formed m-RNA [71].

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CHAPTER 4

THE ISOLATION OF ADRENAL AND LIVER CYT P-450 REDUCTASE

4.1. Introduction

Cyt P-450 reductase was previously isolated from a variety of sources (animals, plants and yeast) and different animal organs [2, 9, 71, 63, 4]. The enzyme has, however, not been purified from ovine tissue. A variety of methods have been published for the isolation and purification of liver and adrenal cyt P-450 reductase [97, 98, 2, 99]. The enzyme has previously been isolated by two different procedures, a protease solubilizing method and a detergent solubilizing method [17, 43, 99].

The protease solubilized cyt P-450 reductase has a lower Mr than the detergent solubilized enzyme due to the loss of the membrane binding segment. This method of isolation only yields the catalytic domain. The role of the membrane binding domain is to maintain the correct orientation of the cyt P-450 reductase during electron transfer. As a result of the loss of the hydrophobic segment, the protease solubilized cyt P-450 reductase is unable to interact with cyt P-450. The enzyme is however able to transfer electrons to artificial electron acceptors such as cyt c [17, 43].

The detergent solubilized cyt P-450 reductase contains both the catalytic and the hydrophobic domains. The enzyme is able to reduce its native electron acceptor cyt P-450 and artificial electron acceptors such as cyt c and cyt b_5 [2, 99, 98]. Several methods for the isolation of cyt P-450 reductase have been published in which Emulgen 913 is used as detergent [97, 98]. The microsomal electron transfer components from various organs in various animals have been

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successfully purified and characterized [31, 56, 40, 2, 37]. The cyt P-450 reductase from bovine adrenal glands for instance, was solubilized with a non-ionic detergent and purified with affinity chromotography [2]. The isolated enzyme has a Mr of 80 000 and contains one mol of FAD and FMN per mol protein [2].

Three detergent solubilization methods were used in this study for the isolation of ovine adrenal cyt P-450 reductase [99, 98, 97]. The procedure used by Estabrook [97] for the isolation of rat liver cyt P-450 reductase consists of solubilization of microsomes in a detergent containing Tris buffer, followed by ionexchange. The ion exchange gel (DE-52) is an anion exchange gel which removes most of the impurities from the microsomal preparation. The cyt P-450 reductase is subsequently purified using affinity chromotography (2'5'ADP-Sepharose 4B), gel fitration and ion exchange on hydroxylapatite. The affinity chromotography step is the major purification step in the isolation. The two subsequent chromotography steps are required to obtain the enzyme in a buffer without 2'AMP or $NADP^+$ (removed by gelfiltration on Sephadex G-200 gel) and detergent (removed by anion exchange on hydroxylapatite). The method described by Yasukochi *et al.* [98] is similar to that of Estabrook [97] except that different buffers are used during purification by this method. Both these methods were used in this study in order to determine which one of the isolation procedures were the more suitable for ovine adrenal cyt P-450 reductase purification.

The previously described isolation methods entails four chromotography steps while a method described by Shephard *et al.* [99] for the isolation of rat liver cyt P-450 reductase contained only one chromotography step. In this method the detergent solubilized preparation is loaded directly onto an affinity chromotography column (agarose-hexane-ADP) without

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partially purifying the enzyme first. The application of the above mentioned three methods for the isolation of ovine adrenal cyt P-450 reductase will subsequently be discussed.

4.2. Preparation of microsomes

Microsomes are used in all the previously described methods as a primary source of cyt P-450 reductase. Therefore microsomes was prepared from the ovine adrenal, ovine liver and bovine adrenal.

4.2.1. The preparation of ovine adrenal microsomes

Microsomes were prepared from adrenal glands collected from freshly slaughtered sheep. After removal of the capsule, the glands were homogenized in a Warring blendor in sucrose solution. This suspension was further homogenized with a motor-driven homogenizer and the homogenate centrifuged at low speed to remove cell debris and mitochondria. The supernant was ultra-centrifuged and the resulting pellet resuspended in a KCl solution and subjected to ultra-centrifugation to remove traces of contaminating hemoglobin [100]. The final microsomal pellet was resuspended in a Tris-sucrose buffer and stored in aliquots in liquid nitrogen.

4.2.2. The preparation of ovine liver microsomes

The liver of a freshly slaughtered sheep was removed and perfused with ice cold saline solution. The liver capsule was subsequently removed and the tissue cut into small pieces which were stored at -70^{o}C. For the preparation of microsomes the frozen perfused liver pieces were treated in the same way as described for ovine adrenal glands.

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4.2.3. The preparation of bovine adrenal microsomes

Bovine adrenal microsomes were prepared using the same procedure described for ovine adrenal microsomes. In this preparation however only the cortex was used for the preparation.

4.3. The isolation of cyt P-450 reductase form ovine adrenal microsomes

The fractions containing the cyt P-450 reductase were identified by assaying for cyt c reductase activity in all the fractions and only pooling the most active fractions. Cytochrome P-450 reductase was assayed as follows: an aliquot of the cyt P-450 reductase preparation was diluted in phosphate buffer. Cytochrome c was added to this solution and this mixture was used as a reference for the spectophotometer. Cytochrome c reduction was initiated by the addition of NADPH and the progress of the reaction was followed by monitoring the increase in absorbance at 550 nm. An example of a typical cyt c reductase assay is shown in Fig. 4.1. A cyt c millimolar absorption coefficient of 29,5 was used in the calculations to determine the initial velocity of the reduction [101].

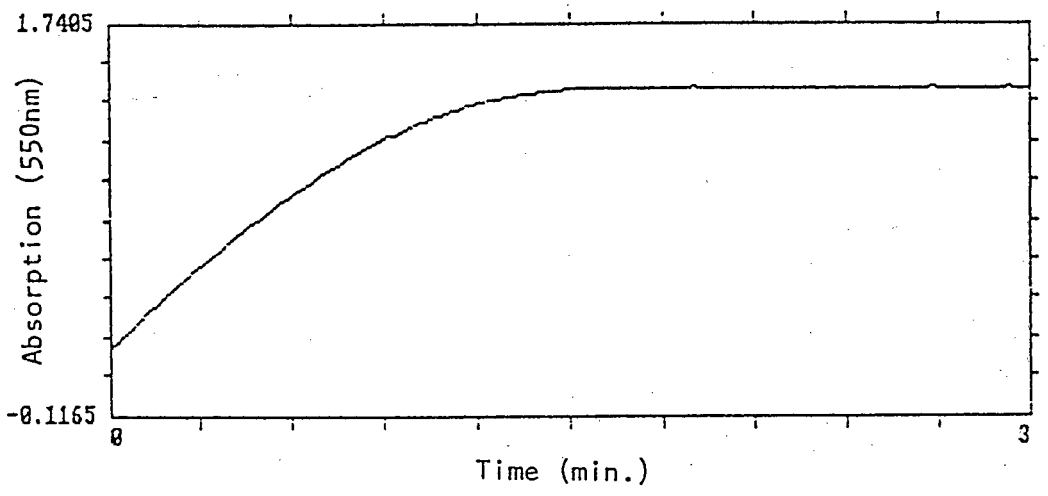

Figure 4.1: Example of a typical cyt c reductase assay.

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An increase in cyt c reductase activity corresponded with an increase in the yellow colour of the cyt P-450 reductase [97]. The purity of the isolated proteins were determined by Soduim dodecyl sulphate poliacrylamide gel electrophoresis (SDS-PAGE).

4.3.1. Procedure 1

This method is a modified version of a procedure decribed by Estabrook [97] for the isolation of rat liver cyt P-450 reductase. Microsomes were diluted 5 fold in a Tris-buffer containing deoxycholate and Triton N101, which solubilized the membrane bound enzymes. The membrane fragments were removed by ultra-centrifugation. A schematic representation of this method is given in Fig. 4.2.

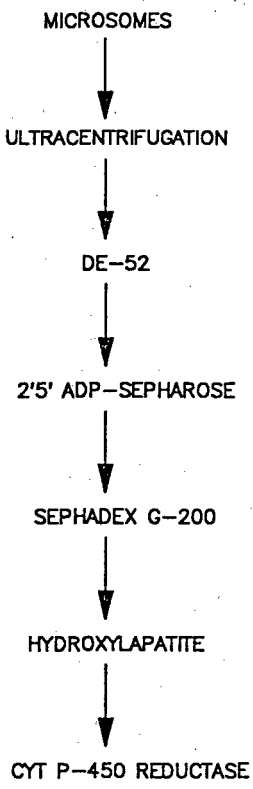

Figure 4.2: Schematic representation of procedure 1

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Anion exchange

The supernatant was poured through cheese cloth, to remove insolubles, and the suspension loaded onto an anion exchange gel (DE-52) packed in a Buchner funnel. The gel was washed twice, first with a Tris-buffer and then with a Tris-buffer containing 0,13 M KCl. The cyt P-450 reductase was subsequently eluted with a Tris-buffer containing 0,3 M KCL.

Affinity chromotography

This fraction was loaded onto a 2'5'ADP-Sepharose 4B column equilibrated with a phosphate buffer. After washing the column with phosphate buffer to remove inactivated proteins the cyt P-450 reductase was eluted with a phosphate buffer containing $NADP^+$. A dilute yellow fraction was obtained which contained the cyt P-450 reductase activity. This solution was concentrated by ultrafiltration.

Gelfiltration

The enzyme preparation was reoxidized with $K_3Fe(CN)_6$ and subsequently subjected to gelfiltration to remove $NADP^+$. The cyt P-450 reductase eluted as a yellow coloured solution from the Sephadex G-200 column. The preparation was again concentrated by ultrafiltration and loaded onto a hydroxylapatite column.

Anion exchange

This column removes the detergent from the enzyme preparation. The hydroxylapatite column was equilibrated and washed with a 20 mM phosphate buffer. The loaded sample was washed until the absorbance of the eluate at 276 nm was less than 0,05. The cyt P-450 reductase was eluted from the hydroxylapatite with a 150 mM phosphate buffer. The purified

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enzyme was subsequently dialyzed overnight against 100 mM phosphate buffer.

This method of isolation was not very successful as the protein yield at the end of the isolation was very low. The protein concentrations of the isolated enzyme preparations were also low and the purity of the enzyme preparations could not be determined by SDS-PAGE. These results could indicate that vast differences between rat liver cyt P-450 reductase and ovine adrenal cyt P-450 reductase exist as the procedure was originally described for rat liver cyt P-450 reductase isolations.

4.3.1.1. The preparation of hydroxylapatite

The hydroxylapatite used for the removal of deterent was prepared by a method which was reported to yield a hydroxylapatite with a high flow rate. The crystalls were prepared by running solutions of $CaCl_2$ and Na_2HPO_4 into a beaker at equal rates. The resulting crystalls were washed several times with water. The precipitate was subsequently diluted with NaOH solution and heated to 100°C. The precipitate was washed several times with water followed by phosphate buffer.

The resulting hydroxylapatite had a very low flow rate which resulted in diffusion of the enzyme preparation on the column. The diffusion of the protein band occurred to such an extent that only a small fraction of the cyt P-450 reductase could be retrieved. The dilution of the enzyme made it difficult to trace the most active fractions resulting in a loss of enzyme. The use of hydroxylapatite proved to be unsuitable for the removal of detergent from this cyt P-450 reductase preparation and other methods for detergent removal were subsequently investigated.

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4.3.2. Procedure 2

In procedure 1 the final step in the purification of the cyt P-450 reductase, the removal of detergent presented problems which led to a loss of protein and a dilute enzyme preparation. In a method of Hiwatashi and Ichikawa [2] DEAE-Sepharose CL 6B was used in a final column in the purification of bovine adrenal cyt P-450 reductase. It was decided to use only this last step in the purification of ovine adrenal cyt P-450 reductase in an attempt to remove the detergent from the protein preparations. A schematic representation of this method is given in Fig. 4.3.

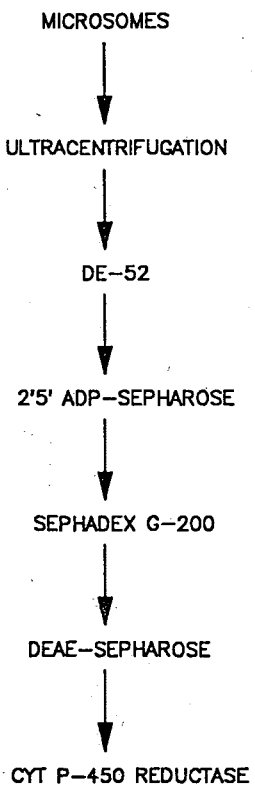

Figure 4.3: Schematic representation of Procedure 2

The hydroxylapatite and the DEAE-Sepharose CL 6B (also an anion exchange gel) column were equilibrated and washed with phosphate buffers. The hydroxylapatite column was eluted with a phosphate buffer having a higher molarity than the

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washing buffer. The DEAE-Sepharose CL 6B was eluted with a phosphate buffer containing deoxycholate and 0,5 M KCl.

The same isolation procedure as described in procedure 1 was followed up to the gelfiltration step which was used to remove the $NADP^+$. The eluate from the gelfiltration was loaded onto a DEAE-Sepharose CL 6B column which was equilibrated with 10 mM phosphate buffer. The column was washed with 50 mM phosphate buffer and the cyt P-450 reductase eluted with the same buffer containing KCL and deoxycholate [2].

The phosphate buffer used to elute the DEAE-Sepharose column contained 0,5 M KCL and 0,1 mM deoxycholate. When both these reagents were solubilized in the phosphate buffer, the viscocity of the buffer was high and could not flow through the column even when pumped with a peristaltic pump. The high viscocity of the buffer caused the protein band to diffuse. In an attempt to elute the enzyme the elution procedure was performed at room temperature where the buffer was less viscose. The yellow coloured cyt P-450 reductase band turned lightbrown after 24 hours at room temperature and it was necessary to transfer the gel to a Buchner funnel and to elute the cyt P-450 reductase with phosphate buffer containing 0,5 M KCl.

It was later found that if the deoxycholate was solubilized completely in distilled water in a sonic bath before mixing with the rest of the reagents this effect did not occur. A SDS-PAGE of the isolated cyt P-450 reductase preparation contained one major band thought to be the cyt P-450 reductase, and at least 4 minor bands (Fig. 4.4).

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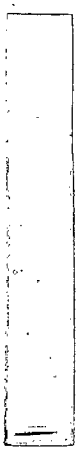

Figure 4.4: SDS-PAGE gel of the cyt P-450 reductase preparation isolated with procedure 2.

4.3.3. Procedure 3

The previously described methods entailed four chromotography steps; anion exchange chromotography, affinity chromotography, gelfiltration and finally ion exchange chromotography. Shephard *et al.* [99] described the isolation of cyt P-450 reductase from pig liver using only one column, an affinity chromotography column. In the original procedure an agarose-hexane-ADP column was used. In this study only 2'5'ADP-Sepharose 4B gel was available and was therefore used in the isolation according to the method published by Shephard *et al.*

Microsomes were suspended to a concentration of 1,9 mg/ml in a potassuim phosphate buffer containing an antioxidant and a protease inhibitor. Cholate was added to the preparation to yield a final cholate concentration of 0,7% ($^{w}/_{v}$). After stirring on ice the preparation was centrifuged and the supernatant diluted with a phosphate buffer to a protein concentration of 1,5 mg/ml. This solution was loaded onto a 2'5'ADP-Sepharose 4B column. The column was washed twice with phosphate buffers containing 300 mM and 30 mM phosphate

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respectively. The cyt P-450 reductase was finally eluted with a 30 mM phosphate buffer containing 1,3 mM $NADP^+$.

In the method published by Shephard *et al.* fractions with a $A_{455}:A_{380}$ ratio of more than one were collected from the agarose-hexane-ADP column. All fractions collected from the 2'5'ADP-Sepharose, however, had absorption ratios of 1 or below. Eventually all fractions containing a yellow coloured solution was pooled. Three major and one minor band was observed on a SDS-PAGE-gel (Fig. 4.5) of the final preparation and it was concluded that the affinity gel reqiured for this isolation was agarose-hexane-ADP and that the 2'5'ADP-Sepharose could not be used as a substitute.

Figure 4.5: SDS-PAGE gel of the cyt P-450 reductase preparation isolated with procedure 3.

4.3.4. Procedure 4

This method is a modified version of a procedure described by Yasukochi *et al.* [98] for the isolation of pig liver cyt P-450 reductase, and was found to most suitable for the isolation of ovine adrenal cyt P-450 reductase. For this method the same chromotography columns as procedure 1 was used. In these two methods, procedure 1 and procedure 4 the sequence of the columns were the same but different buffers

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were used. Microsomes were suspended in a Triton N101 containing Tris-HCl buffer and ultra-centrifuged to remove membrane fragments. DE-52 was poured in a Buchner funnel and equilibrated with a Tris-HCl buffer. The supernatant was loaded onto the gel and the gel was washed with phosphate buffer followed by phosphate buffer containing 0,12 M KCl in fractions of 250 ml. The cyt P-450 reductase was subsequently eluted with a phosphate buffer containing 0,3 M KCL in fractions of 250 ml. An elution profile for cyt P-450 reductase from DE-52 is shown in Fig. 4.6. Fractions 1-4 was eluted with the buffer containing 0,12 M KCL and fractions 5-8 was eluted with the buffer containing 0,3 M KCL.

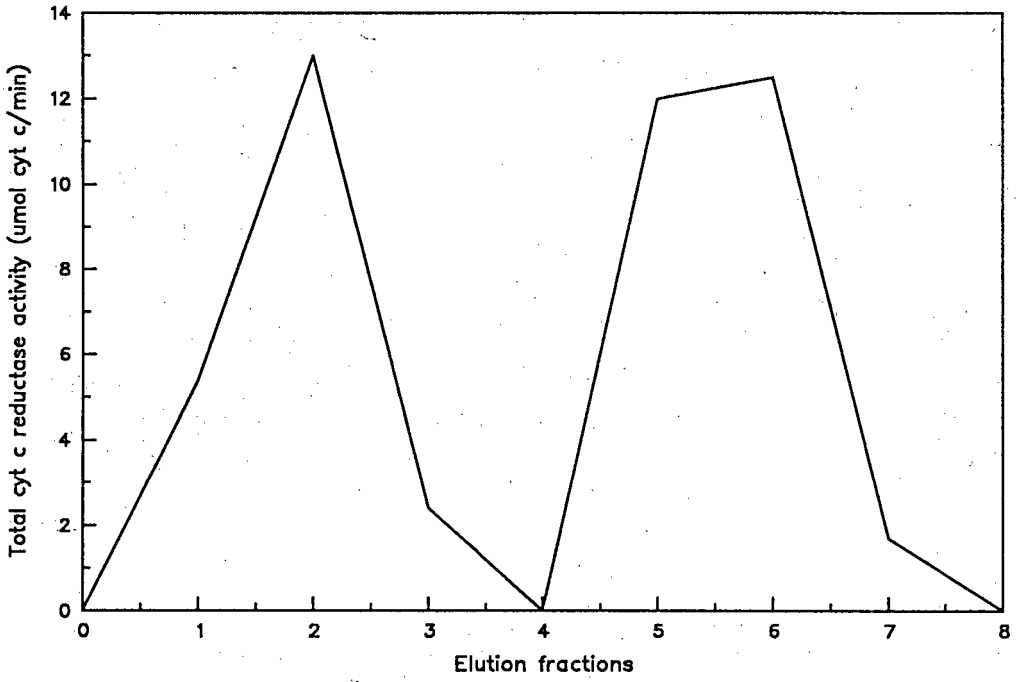

Figure 4.6: Activity profile of cyt P-450 reductase from DE-52 batch exchange column.

The cyt P-450 reductase containing preparation was diluted with an equal volume of glycerine (20%) and applied to a 2'5'ADP-Sepharose column previously equilibrated with phosphate buffer. The column was washed with phosphate buffer and the cyt P-450 reductase finally eluted with a

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phosphate buffer containing 1,2 M $NADP^+$. The cyt P-450 reductase containing fractions were pooled and concentrated. Subsequently the enzyme preparation was reoxidized with $K_3Fe(CN)_6$ and loaded onto a Sephadex G-200 column. The enzyme was eluted with phosphate buffer as a yellow coloured fraction. This fraction was applied to a hydroxylapatite column. The column was washed with a phosphate buffer until the absorbance of the eluate at 280 nm was less than 0,02. The enzyme was eluted with a phosphate buffer with a higher molarity than the washing buffer. The resulting cyt P-450 reductase preparation (Red 1) was concentrated and dialyzed overnight against the elution buffer containing EDTA. SDS-PAGE analysis of Red 1 showed that the preparation consisted of two fractions; a major band corresponding to a Mr of 78 000 and a minor band corresponding to a Mr of 73 500 (Fig. 4.7). This preparation was, however, the purest obtained thusfar.

Figure 4.7: SDS-PAGE gel of Red 1

In order to obtain a pure cyt P-450 reductase preparation, procedure 4 was slightly modified as follows. It was found that $NADP^+$, used in the elution buffer inhibited cyt c reduction by Red 1. When 2'AMP was used instead of $NADP^+$ in the elution buffer, no further interference was observed.

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Emulgen 913, previously unavailable, subsequently became available, and was used as detergent in the place of Triton N101. The isolation procedure was followed exactly as decribed above up to the elution step from the 2'5'ADP-Sepharose. An elution profile of cyt P-450 reductase from 2'5'ADP-Sepharose is shown in Fig. 4.8. The cyt P-450 reductase containing fractions were pooled and concentrated by ultrafiltration.

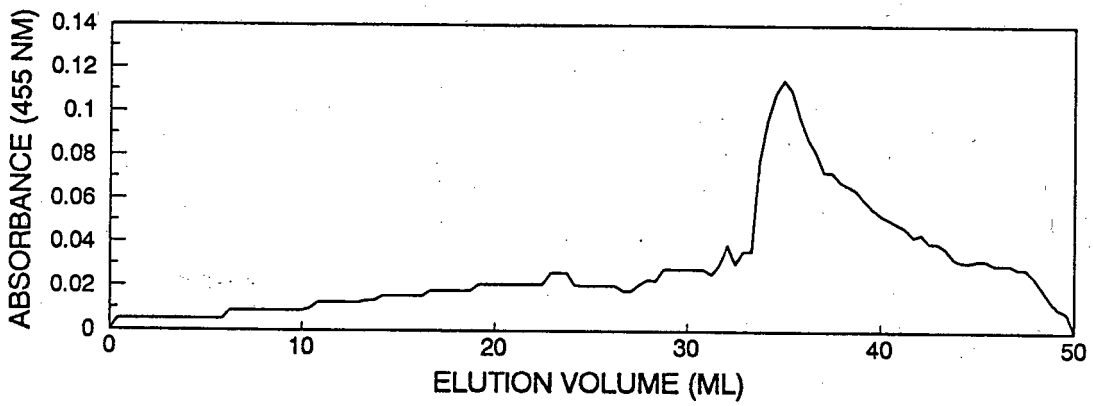

Figure 4.8: Elution profile of cyt P-450 reductase (Red 2) from 2'5'ADP-Sepharose column. Cytochrome P-450 reductase absorbs maximally at 455 nm [102].

The resulting isolated enzyme preparation, refered to as Red 2, showed a single band on a SDS-PAGE gel corresponding to a Mr of 78 000 (Fig. 4.9).

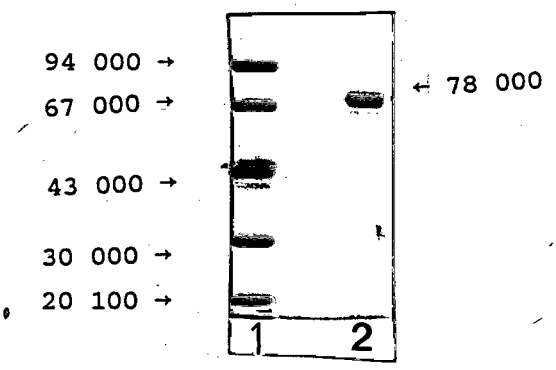

Figure 4.9: SDS-PAGE gel of Mr markers (lane 1) and Red 2 (lane 2).

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4.4. Characterization of Red 1 and Red 2

4.4.1. Isoelectric focussing of the cyt P-450 reductase preparations

Isoelectric focussing showed two bands for both the ovine adrenal cyt P-450 reductases, Red 1 and Red 2. A major band at pH 5,3 and a minor band at pH 5 was observed on the gel (Fig. 4.10).

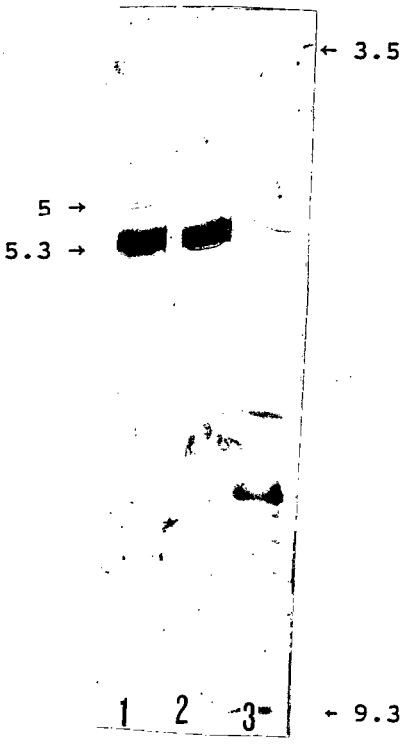

Figure 4.10: Isoelectric focussing of Red 1 (lane 1), Red 2 (lane 2) and pH markers (lane 3).

4.4.2. Spectral properties

An optical absorpsion spectrum of Red 2 is shown in Fig. 4.11. Red 2 as well as all spectra of pure ovine adrenal cyt P-450 reductase isolated later showed identical absorption maxima at 380 nm and 455 nm with shoulders at 360 nm and 480 nm. Identical spectral results were reported

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for the bovine adrenal cyt P-450 reductase isolated earlier [2].

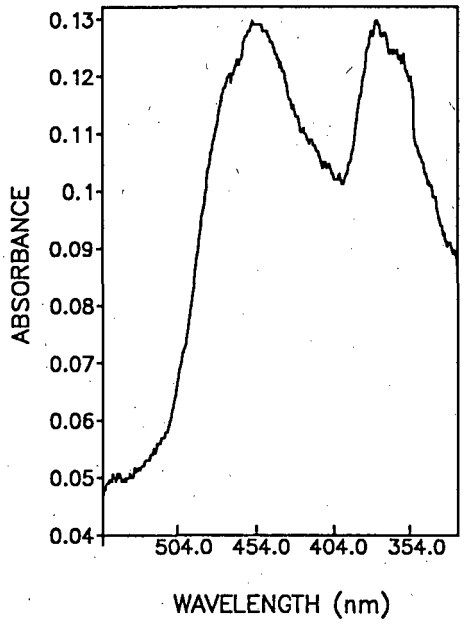

Figure 4.11: Absorption spectrum of Red 2 (protein concentration = 1,32 mg/ml).

4.4.3. Cytochrome c reductase activity of Red 1 and Red 2

The cyt c reductase activity of Red 1, Red 2 and Red 2a are shown in Table 1 in comparison to bovine cyt P-450 reductase previously isolated [2]. Red 2 was found to be the most active of the isolated cyt P-450 reductases.

4.4.4. Temperature stability

After storage of Red 2 at -20^{o}C and thawing and freezing a minor band appeared in the same region as the minor band found in Red 1 (Fig. 4.12). This second band is likely to be a degradation product of the pure enzyme due to temperature

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lability of the protein or because of the freezing and thawing. To discriminate between the two forms of Red 2, the minor form will be refered to as Red 2a.

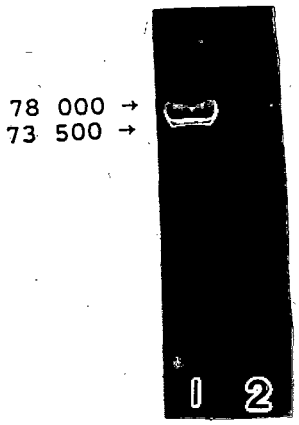

Figure 4.12: SDS-PAGE gel of Red 2a (lane 1) and Red 1 (lane 2).

Red 1 and Red 2 was subsequently used as antigens in the immunization of rabbits.

4.5. The isolation of cyt P-450 reductase from ovine liver

Ovine liver cyt P-450 reductase was partially purified and used in comparitive immunochemical studies. Ovine liver microsomes, prepared as described earlier, were used for the isolation of the cyt P-450 reductase. The method of Yasukochi *et al.* [98] was used as described for the isolation of Red 2a (procedure 4).

The cyt P-450 reductase preparation from ovine liver was not pure as a SDS-PAGE gel showed one protein band with a Mr corresponding with the that of the ovine adrenal cyt P-450 reductase (78 000), and at least 3 other contaminating protein bands (Fig. 4.13). It was not attempted to purify these preparations any further as the immunochemical studies

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it was intended for did not require a purified liver cyt P-450 reductase as they were only used in comparitive experiments.

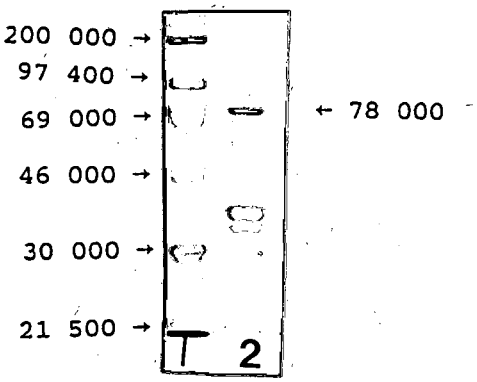

Figure 4.13: SDS gel of Mr markers (lane 1) and the enzyme preparation of ovine liver cyt P-450 reductase isolated by procedure 4 (lane 2).

The cyt c reductase activity of the ovine liver cyt P-450 reductase preparation is shown in Table 1 in comparison to the activities of the other isolated cyt P-450 reductases.

4.6. The isolation of cyt P-450 reductase from bovine adrenals

Microsomes, prepared as described earlier, were used for the isolation of cyt P-450 reductase from bovine adrenals. The modified method of Yasukochi *et al.* was used for this isolation as described earlier for ovine adrenal cyt P-450 reductase. The resulting purified enzyme showed one major protein band on SDS-PAGE gel corresponding to a Mr of 78 000 and two minor bands (Fig. 4.14).

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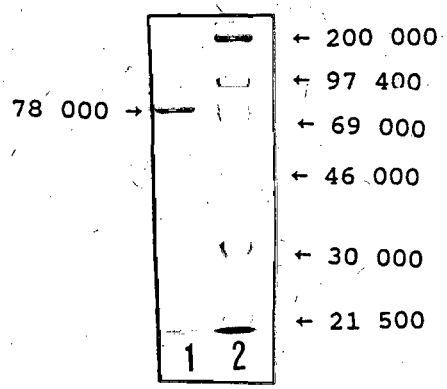

Figure 4.14: SDS gel of bovine adrenal cyt P-450 reductase isolated with procedure 4 (lane 1) and Mr markers (lane 2).

All cyt P-450 reductases isolated were assayed for cyt c reductase activity. In Table 1 the cyt c reductase activity of Red 1, Red 2, Red 2a, the ovine liver (Red OL) and bovine adrenal cyt P-450 reductase (Red BA) is shown and compared to results reported earlier for bovine adrenal cyt P-450 reductase [2]

Table 1: Cyt c reductase activities of the isolated cyt P-450 reductases.

Cyt P-450 reductase	Specific activity (μmol/min/mg protein)
Red 1	1,98
Red 2	11,77
Red 2a	2,5
Red OL	1,25
Red BA[a]	1,0
Red BA[b]	29,5

[a] Bovine adrenal cyt P-450 reductase isolated
[b] Bovine adrenal cyt P-450 reductase isolated by Hiwatashi and Ichikawa [2]

The cyt c reductase activity of the pure cyt P-450 reductases were noticably lower than that reported earlier

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for bovine adrenal cyt P-450 reductase. These lower values of the bovine adrenal and ovine liver enzymes can be attributed to impurities in the enzyme preparations. There was a 5 fold decrease in the cyt c reductase activity of the degraded Red 2a preparation.

4.7. Discussion

Microsomes were prepared using a method developed by Estabrook [97]. The protein concentration of the microsomes varied between 25-40 mg/ml depending on how much Tris-sucrose was used in the final step of the preparation. Microsomes prepared from 100 g of adrenal glands or liver tissue had an approximate protein content of 1 g.

Three detergent solubilizing isolation methods were used of which only one was successful for the purification of ovine adrenal cyt P-450 reductase. It was not possible to use the methods described by Shephard *et al.* [99] and Estabrook [97] for the purification of the ovine adrenal cyt P-450 reductase. The reason for this ineffective isolations may be related to the detergent used and problems encountered with the hydroxylapatite column. In both of the original methods Emulgen 913 was used but was unavailable at that time, it was subtituted with Triton N101. With the use of Emulgen 913 in the method of Yasukochi *et al.* the cyt P-450 reductase yield increased and the purity of the protein preparations increased. These results indicate that Triton N101 cannot substitute Emulgen 913 for the isolation of cyt P-450 reductase from ovine adrenals.

The ovine adrenal cyt P-450 reductase was purified to apparent homogenity. The Mr of the cyt P-450 reductases was 78 000, which corresponded well with the Mr of bovine

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adrenal cyt P-450 reductase previously estimated at 80 000 [2]. The Mr of the minor protein band in Red 1 was 73 500.

This minor band is a degradation product of the pure cyt P-450 reductase due to the temperature lability of the enzyme. This idea was supported by the fact that the homogeneous Red 2 degraded and formed Red 2a, which consisted of two proteins identical to those found in Red 1. The newly formed protein had a Mr of 73 500, which corresponded with the Mr found for the minor protein band in Red 1.

The activities of the isolated proteins Red 1, Red 2 and Red 2a were respectively 1,98, 11,77 and 2,5 µmol cyt c/min/mg protein. These values are much lower than that reported earlier for the bovine adrenal cyt P-450 reductase. The reason for this could be the degradation of the protein or the isolation method used for the purification. Red 1, Red 2a, Red OL and Red BA showed partial degradation in different proportions as they all contained a fragment corresponding to the Mr 73 500 of the degradation product. The protein fragment lost during the degradation process had an Mr of 4 500. Cytochrome c reductase activity decreased with the formation of this fragment. Protease treated cyt P-450 reductase produced an enzyme which contained no membrane binding segment but retained the ability to reduce cyt c. It is clear that the fragment removed during degradation is not the membrane binding segment of the cyt P-450 reductase molecule, but a protein fragment essential for enzymatic activity [17, 43]

Upon isoelectric focussing Red 1 and Red 2 both showed a major band at pH 5 and a minor band at pH 5,3. These results correspond well with the results found by Guengerich [96] for homogeneous species of rabbit and rat cyt P-450 reductases. The rabbit lung and liver cyt P-450 reductase

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gave two major bands at pI 5,1 and 5,2 while the rat liver enzyme gave a number of bands in the range of 4,7-5,4.

The absorption spectrum of Red 2 indicates that the protein isolated was indeed a flavoprotein, having characteristic absorption maxima at 380 nm and 455 nm. This typical absorption maxima was previously found for adrenal [2] and liver [20] cyt P-450 reductases. From these results and the comparison to other cyt P-450 reductase characteristics it is concluded that the ovine adrenal enzyme purified in this study was cyt P-450 reductase.

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CHAPTER 5

THE PREPARATION OF ANTIBODIES AGAINST OVINE ADRENAL CYT P-450 REDUCTASE

5.1. Introduction

The production of antibodies to protein antigens using conventional immunization protocols usually requires relatively large amounts of proteins which are not always available. For this reason a method of antigen preparation which requires less protein, but still results in an immune response with high antibody levels was developed [13]. In this method the protein to which antibodies are required is adsorbed to so-called naked bacteria (NB) and such complexes are then used to immunize experimental animals.

The bacterial strain used for the production of naked bacteria is *Salmonella minnesota R595* [13]. The natural antigenic determinants of the bacterial cell wall of these bacteria is removed by mild acid treatment. This resulted in a hydrophobic outer surface to which antigens such as proteins could be adsorbed. The resulting complexes are then used to raise antibodies against these grafted antigenic determinants. This method of antigen preparation has been used previously to raise antibodies against lipopolysaccharides, glycoproteins and proteins [11, 12, 13].

In this study ovine adrenal cyt P-450 reductase was isolated with a yield of approximately 1 mg protein. It was thus decided to use NB as antigen carriers for the preparation of antibodies against this enzyme. The isolated ovine adrenal cyt P-450 reductase was adsorbed to NB and these complexes were used to immunize rabbits according to the method described by Bellstedt *et al* [13].

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Modern immunodetection procedures such as the enzyme-linked immunosorbent assay (ELISA) [103] and western blots [104] are much more sensitive and more specific than conventional detection methods such as SDS-PAGE. ELISA is an enzyme immunodetection system in which the antibody or the antigen is immobilized on a solid phase. In this study the antigen (cyt P-450 reductase) was adsorbed to the surface of ELISA plates. The primary antibody binds to the immobilized antigen. The primary antibody in turn is recognized by a bridge antibody, which amplifies the system as several bridge antibodies can bind to a single primary antibody. The bridge antibody is recognized by an antibody complexed with enzyme molecules, which form the recognition system of the ELISA. This complexed enzyme reacts with a substrate to produce a soluble product the concentration of which can be measured spectrophotometrically. The detectability of this system is increased, due to the amplification with bridge antibodies and due to the presence of the antibody enzyme complex [103].

Western blotting, also called immunoblotting is performed on nitrocellulose membranes [104]. The antigen (cyt P-450 reductase) is first separated by SDS-PAGE and electrotransferred to the nitrocellulose membrane. The immunoblotting steps used in this study were the same as described for the ELISA. The substrate used in the immunoblotting procedure produces a precipitating product. Immunoblots can be used to detect very small amounts of proteins, as well as a specific protein in a crude preparation. In this study, immunoblots were used to indicate the presence of cyt P-450 reductase in certain cellular fractions as well as the structural relationship of ovine and bovine cyt P-450 reductase. The ovine adrenal cyt P-450 reductase was subjected to temperature lability tests and the antibodies were used to detect the degradation fragments. In this study immunoblotting was also used to

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determine the relationship between the microsomal cyt P-450 reductase isolated from ovine and bovine adrenals, ovine liver as well as the mitochondrial ADXR from ovine adrenals.

5.2. The adsorption of cyt P-450 reductase to NB

Two preparations of cyt P-450 reductase (Red 1 and Red 2, see Chapter 4) were adsorbed to NB respectively and the resulting complexes were used to elicit immune responses in rabbits [13]. To this end a suspension of NB in distilled water was prepared by homogenization with a loosely fitting Teflon homogenizer. Subsequently the NB suspension and the protein solution (Red 1 and Red 2, respectively) were mixed so that the ratio by dry mass of protein to NB was 1:5. The suspension was dried with rotary evaporation and the resulting complexes resuspended by brief sonication in PBS to the desired concentration.

5.3. The preparation of antibodies against cyt P-450 reductase

Rabbits were immunized over a period of 39 days. The first two injections were given on day 1 and 4 with 0,25 and 0,5 ml respectively of a 120 μg protein-NB complex/0,5 ml suspension. Further injections were given on days 7, 11, 18, 21, 25, 32, 35 and 39 with 0,5 ml of a 240 μg protein-NB complex/0,5 ml suspension. A small volume of blood (2 ml) was drawn on days 0, 11, 18, 25, 32 and 35 and a large volume (20 ml) on day 46. The antisera were prepared by allowing the blood to clot and serum was subsequently isolated by centrifugation and stored at -20^{o}C for further studies.

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5.4. Enzyme-linked immunosorbent assay for cyt P-450 reductase antibodies

The levels of the antibodies against cyt P-450 reductase during the immunization of rabbits were determined with ELISA. ELISA plates were coated with cyt P-450 reductase diluted (1 μg/ml) in bicarbonate buffer overnight at 4°C. The coating solution was then decanted and non-specific binding was blocked by incubation with casein-bicarbonate buffer after which the plates were washed 3 times with PBS-Tween. This washing step was included after each of the subsequent steps. A dilution series of antiserum was made in casein buffer, starting with a dilution of 1 in 20 (wells 2-11). Well 1 was coated with the cyt P450 reductase but was not incubated with anti-cyt P-450 reductase antibodies. In its place only casein buffer was used. Well 12 was not coated with the cyt P-450 reductase but was incubated with a 1 in 20 dilution of anti-cyt P-450 reductase antibodies. An incubation period of 1 hour at 37°C followed. These two wells (1 and 12) were used as controls to determine whether the casein buffer (well 1) or the anti cyt P-450 antibodies (well 12) showed any non-specific binding to the ELISA plate. The rest of the steps were the same for all the wells. To this end, diluted sheep-anti-rabbit antibodies (1 in 400) were pipetted into all wells and the plates were incubated at 37°C for 1 hour. Suitably diluted rabbit-peroxidase-antiperoxidase complex (1 in 10 000) was subsequently added and the plates incubated at 37°C for 1 hour. After a final washing step the substrate solution was added and the absorbance at 405 nm recorded after 10 minutes. The substrate solution contained ABTS (2,2'azino-di[3 ethylbenzthiazoline-sulfonic acid (6)]) and H_2O_2 in citrate buffer (0,1 M, pH 5).

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The immune response patterns of the two rabbits immunized with Red 1 (rabbit 1) and Red 2 (rabbit 2) are shown in Fig. 5.1.

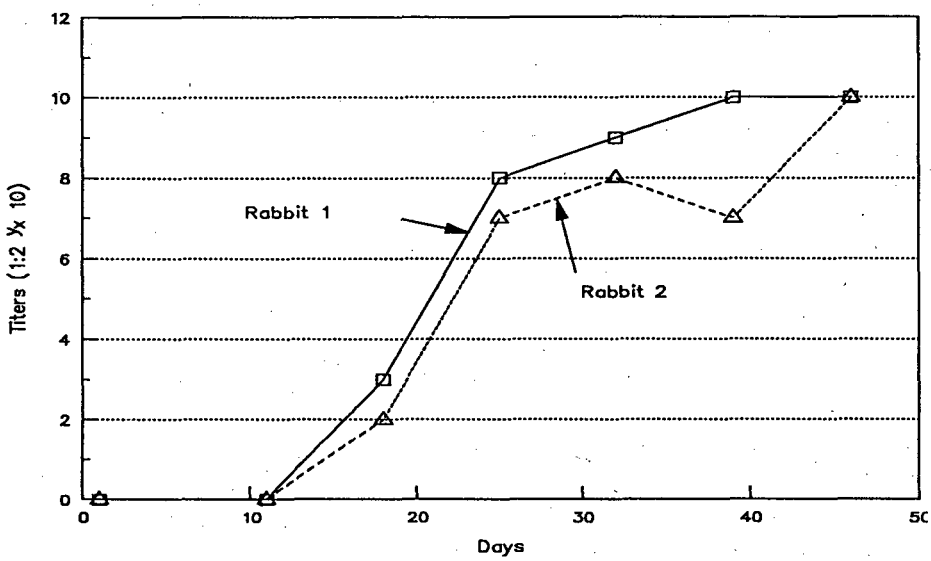

Figure 5.1: The immune response patterns of rabbit 1 (immunized with Red 1) and rabbit 2 (immunized with Red 2) as determined with an ELISA.

5.5. Electroblotting

The antibodies raised in this study against Red 1 and Red 2 were employed in immunoblotting experiments to determine whether they could be used to detect isolated cyt P-450 reductases purified from ovine liver, ovine and bovine adrenals and in crude preparations (microsomes) from these sources.

The isolated cyt P-450 reductases (bovine and ovine) and the microsomes were first separated on a SDS-PAGE gel as described in the previous chapter. The proteins were then transferred from the SDS-PAGE-gel to a nitrocellulose membrane using an electro-transfer procedure. The immunoblotting procedure was subsequently performed on this membrane [104].

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5.5.1. Electro-transfer

The separated proteins were transferred from the SDS-PAGE-gel to a nitrocellulose membrane by electro-transfer [104]. A water saturated nitrocellulose membrane was placed on one side of the gel. The gel and membrane was sandwiched between scouring pads and blotting paper. A perspex grid was placed on either sides of the sandwich, which was inserted into a tank of Tris-buffer. A constant current was applied across the gel overnight. This resulted in an electrophoretic transfer of proteins from the SDS-PAGE-gel to the nitrocellulose membrane.

5.5.2. Immunodetection procedures

Following the transfer of the proteins to the nitrocellulose membrane, it was blotted dry and non-specific binding was blocked by incubation with casein buffer. Two membranes were prepared in this way (with Red 1, Red 2 and ovine adrenal microsomes) and subsequently incubated at 37^{o}C for 2 hours with an optimal dilution of the rabbit-anti-cyt P-450 reductase antiserum (anti-Red 1 and anti-Red 2 respectively both at 1 in 4 000 dilution). The membranes were washed 4 times after this step and after all subsequent incubation steps with PBS-Tween. Therefore the membranes were incubated at 37^{o}C for 1 hour with sheep-anti-rabbit antibodies (1 in 200). Subsequently the membranes were incubated with rabbit-PAP complex (1 in 5 000) at 37^{o}C for 1 hour. After a final washing step the membranes were incubated with substrate. The substrate solution was prepared by dissolving 4-chloro-naphtol in methanol after which this solution was added to PBS and H_2O_2. The membrane was washed with distilled water and blotted dry. At the position on the nitrocellulose membrane where anti-cyt P-450 reductase antibodies detected a protein the substrate formed a blue precipitate.

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The antibodies against Red 1 and Red 2 detected Red 2 and both the bands of Red 1 and the reductase in ovine adrenal microsomes respectively (Fig. 5.2 and Fig. 5.3).

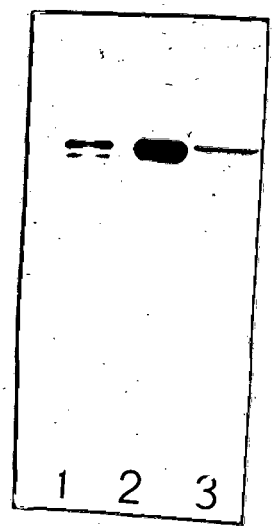

Figure 5.2: Immunoblot of Red 1 (lane 1, 1,5 μg), Red 2 (lane 2, 1,5 μg) and ovine adrenal microsomes (lane 3, 18 μg) detected with anti-Red 1-antibodies.

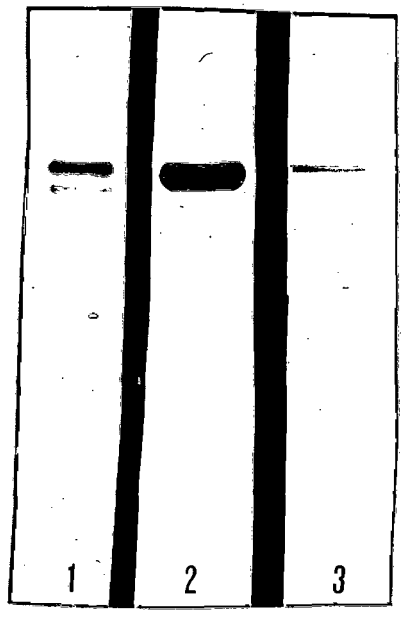

Figure 5.3: Immunoblot of Red 1 (lane 1, 1,5 μg), Red 2 (lane 2, 1,5 μg) and ovine adrenal microsomes (lane 3, 18 μg) detected with anti-Red 2-antibodies.

Antibodies to Red 2 were unable to detect the ADXR found in ovine adrenal mitochondria (Fig. 5.4). Antibodies against

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the ADXR [105] did not recognize the microsomal reductase (Fig. 5.5).

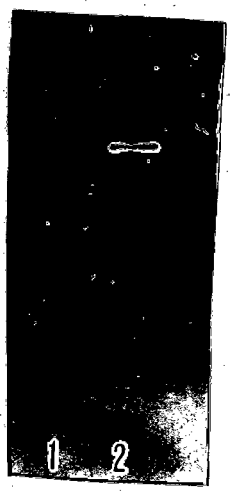

Figure 5.4: Immunoblot of ovine adrenal mitochondrial ADXR (lane 1, 3 μg) and the ovine adrenal microsomal Red 1 (lane 2, 1 μg) detected with anti Red 1-antibodies.

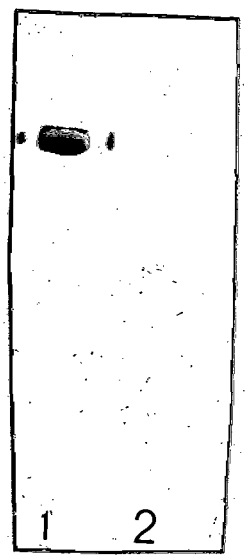

Figure 5.5: Immunoblot of ovine mitochondrial ADXR (lane 1, 1 μg) and the ovine adrenal microsomal Red 1 (lane 2, 3 μg) detected with anti adrenodoxin reductase-antibodies.

Immunoblotting of Red 2, partially purified ovine liver and bovine adrenal cyt P-450 reductase with the antibodies against Red 2 is shown in Fig. 5.6. The antiserum showed a

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strong band with its homologous antigen Red 2 (Fig. 5.6, lane 1) and showed weaker bands with ovine liver reductase (lane 2) and bovine adrenal reductase (lane 3). These proteins were applied at identical concentrations to Red 2. In lane 4 bovine adrenal reductase was applied at double the concentration of Red 2. These results show that anti-Red 2 antibodies crossreact weakly with ovine liver and even more weakly with bovine adrenal reductase.

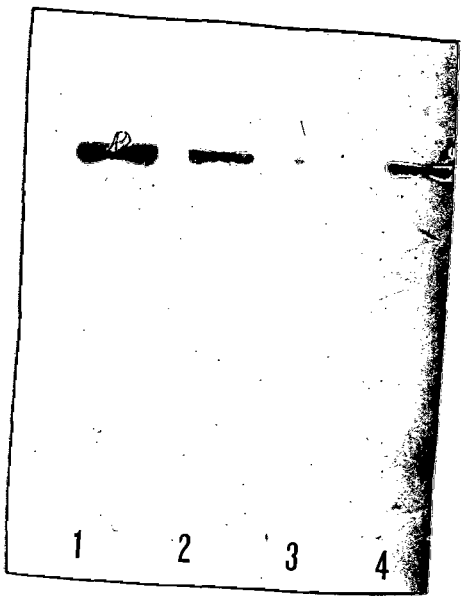

Figure 5.6: Immunoblot of Red 2 (lane 1, 0,5 μg), ovine liver cyt P-450 reductase (lane 2, 0,5 μg) and bovine adrenal cyt P-450 reductase (lane, 0,5 μg) and bovine adrenal cyt P-450 reductase (lane 4, 1 μg) detected with anti-Red 2-antibodies.

Anti-Red 2 antibodies were able to detect the cyt P-450 reductase in microsomal preparations of ovine liver but not in bovine adrenal microsomes (Fig. 5.7) even when the preparations were applied at higher concentrations.

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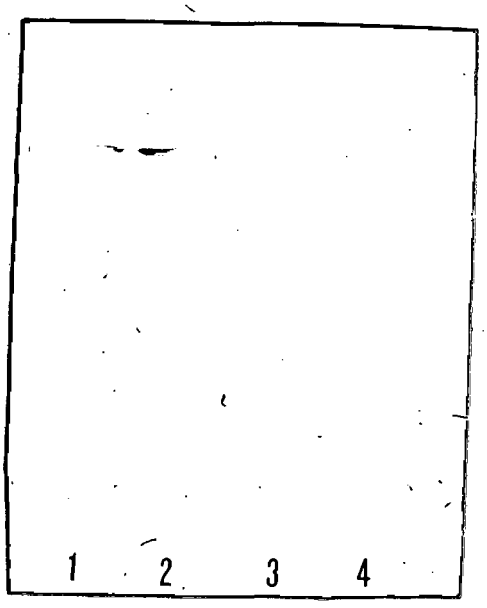

Figure 5.7: Immunoblot of microsomal preparations of ovine adrenals, ovine liver and bovine adrenals (lanes 1-3, 30 µg) and bovine adrenals (lane 4, 60 µg) detected with anti-Red 2 antibodies.

Red 2 showed a single protein band on a SDS-PAGE gel (see chapter 4) as well as on a immunoblot (Fig. 5.3.). After thawing and freezing the protein it was found to contain a second band in the same region as the smaller fragment of Red 1 (Fig. 5.8.).

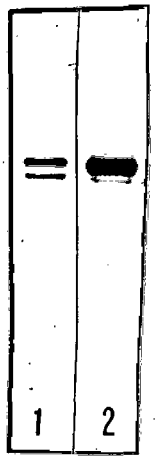

Figure 5.8: Immunoblot of Red 1 (lane 1) and Red 2 (lane 2) detected with anti-Red 1-antibodies after thawing and freezing of Red 2.

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It therefore became apparent that this enzyme was sensitive to higher temperatures The heat lability of the cyt P-450 reductase was therefore investigated further by incubation of the purified enzyme at room temperature (25°C), 37°C and 50°C for 24 hours and 1 week respectively. The results obtained are shown in Fig. 5.9.

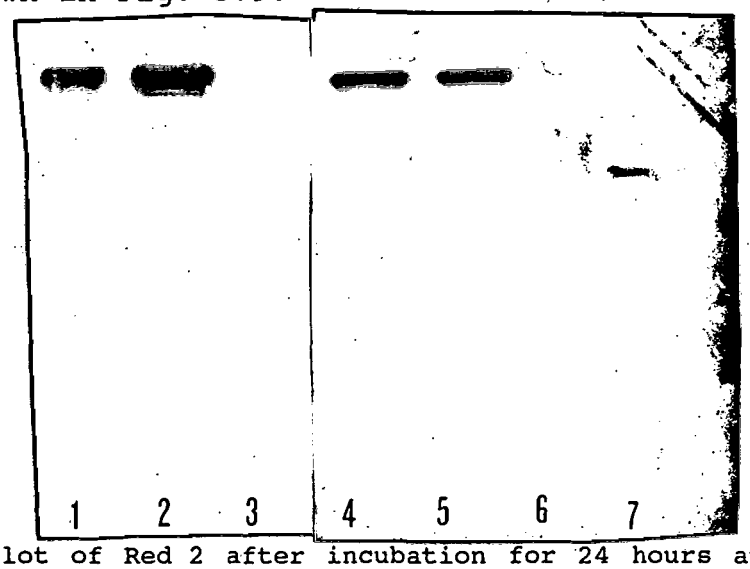

Figure 5.9: Immunoblot of Red 2 after incubation for 24 hours at room temperature (lane 1), 37°C (lane 2) and 50°C (lane 3) and after 1 week at room temperature (lane 5), 37°C (lane 6) and 50°C (lane 7) illustrating the heat lability of Red 2. A control was included in lane 4 and the proteins were detected with anti-Red 2-antibodies.

After 24 hours the enzyme incubated at room temperature showed no degradation (lane 1) while the enzyme at 37°C showed the initiation of the degradation process (lane 2). The enzyme incubated at 50°C denatured completely and showed no bands on the immunoblot (lane 3). After incubation of 1 week the enzyme incubated at 37°C and 50°C denatured completely (lane 6 and 7). Surprisingly the reductase incubated at 25°C showed only slight degradation with the appearance of a second band (lane 5) with a Mr smaller than that of the purified cyt P-450 reductase (lane 4).

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5.6. Discussion

An immune response was elicited in rabbits after immunization with cyt P-450 reductase-NB complexes. The maximum antibody levels were found on day 46 with a titer of 1:10 240 in both rabbits. It was thought at first that Red 1 was not pure as a second protein band was observed on the SDS-PAGE-gels. This second protein band was, however, also recognized by antisera against pure cyt P-450 reductase (Red 2). These results indicate that Red 1 does not contain a contaminating smaller band, but that this protein band derives from the purified reductase itself. It is thus suggested that this second band, with Mr 73 000, is a degradation fragment.

This hypothesis is confirmed by the results obtained in the heat lability study performed on Red 2 using immunoblotting studies. Here it was also found that the incubation at higher temperatures appeared to result in the production of the above mentioned breakdown product of Red 2. It was found that Red 2 was stable for 1 week at 25^{o}C. Higher temperatures however resulted in total degradation of Red 2. As mentioned in chapter 4 the degradation of Red 2 to Red 2a resulted in a 5 fold decrease in cyt c reductase activity of the enzyme. It can be concluded that the degradation of the cyt P-450 reductase with a Mr of 78 000 to a product with a Mr of 73 500 leads to a decrease in cyt c reductase activity of the enzyme.

The anti-Red 2 antiserum could detect both ovine liver and adrenal cyt P-450 reductases equally well. It therefore appears that ovine liver and adrenal cyt P-450 reductases are strongly related and possibly identical. The bovine adrenal cyt P-450 reductase was recognized by the anti-Red 2 to a lesser extent indicating a certain amount of cross-

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reactivity with this enzyme. These results indicate that the bovine and ovine enzymes are immunochemically related.

Cross-reactivity between antibodies against the ovine adrenal cyt P-450 reductase, and the bovine adrenal enzyme is expected to be weak as the two species are totally different. The fact that cross-reactivity does exist indicates that the two species may share similar or nearly similar antigenic determinants.

An immunoblot with microsomal preparations with ovine liver and ovine and bovine adrenals showed that cyt P-450 reductase could only be detected in the two ovine preparations. The reason for this phenomenon could be that the concentration of the bovine enzyme is much lower in the microsomes than the ovine adrenal cyt P-450 reductase. These results with the microsomes correspond with those found with the isolated enzymes. These results could also indicate that the ovine adrenal and ovine liver cyt P-450 reductases are not synthesized as larger precursor molecules and have the same Mr as the isolated enzyme. It was earlier reported that the rat liver cyt P-450 reductase is synthesized in a matured form and not as a precursor [10].

De Duve *et al.* [20] indicated that the electron transport system of the microsomes differs from that in the mitochondria. These conclusions was confirmed by results obtained in this study. Antibodies against the ovine adrenal cyt P-450 reductase did not detect the ovine adrenal mitochondrial reductase, ADXR. Masters *et al.* [31] showed in their studies that the flavoproteins involved in the electron transfer systems of the mitochondria and microsomes of the adrenocortical cells differ immunochemically. Anti-adrenodoxin antibodies had no effect on the electron transport rate of the microsomal system. The microsomal

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flavoprotein was unable to substitute for the mitochondrial flavoprotein in reconstituted systems [31].

Results obtained in these immunochemical studies suggest that the antibodies raised against ovine adrenal cyt P-450 reductase can be used in comparitive studies with other ovine organs. Cross-reactivity with bovine adrenal cyt P-450 reductase was weak and indicates a high specificity of the antibody towards the antigen (ovine adrenal cyt P-450 reductase).

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CHAPTER 6

DISCUSSION

Cytochrome P-450 reductase is a membrane bound flavoprotein found in animals, yeasts and plants species [21, 25, 27, 3]. In animals the enzyme is found in various organs such as kidneys, adrenals, lungs and liver [2, 3, 19, 20]. Cytochrome P-450 reductase forms an integral part of the cyt P-450 hydroxylation system. These cyt P-450 systems are involved in numerous metabolic pathways such as the metabolism of steroids, drugs, and carcinogens [28, 29, 30]. In the adrenal gland the cyt P-450 hydroxylation system is involved in the metabolism of steroid hormones [1].

Cytochrome P-450 reductase has previously been isolated from various mammalian organs. It was, however, never before isolated from ovine tissue. The procedures investigated for the isolation of this enzyme was modified slightly for the isolation of the ovine adrenal cyt P-450 reductase. Three different methods, were used previously described by Estabrook [99], Shephard *et al.* [101] and Yasukochi *et al.* [100], were used in this study. A modified version of the method described by Yasukochi *et al.* [100] for the isolation of pig liver cyt P-450 reductase was found to be most suitable for the isolation of ovine adrenal cyt P-450 reductase.

This method of isolation resulted in the purification of two cyt P-450 reductases; Red 1 and Red 2. Red 1 showed two protein bands (a major and a minor band) on SDS-PAGE while Red 2 showed only one band. The Mr of Red 2 and the major band of Red 1 was 78 000 and the minor band of Red 1, 73 500.

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The specific activity of Red 2 is six times higher than that found for Red 1. When the single protein band of Red 2 partially degraded to two protein bands, Red 2a, a 5 fold decrease in cyt c reductase activity was found. It can be concluded that the minor band in both these preparations (Red 1 and Red 2a) is enzymatically inactive.

The preparation of antibodies against ovine adrenal cyt P-450 reductase for the use in immunochemical studies was one of the aims of this study. A relatively new technique for the eliciting of an immune response in rabbits was investigated. The adsorption of antigens to NB to form antigen-NB-complexes was performed earlier with lipopolisaccharides, proteins and glucoproteins as antigens [104, 105, 106]. This method uses only 480 ug of protein for one immunization schedule of 39 days.

Red 1 and Red 2 were both used for the production of antibodies in rabbits. The titers of the antibodies were as high as 2560 (anti-Red 1) after only 25 days while after 46 days the antibody titers were 10240 (both antisera) . This serum was used in immunoblotting procedures.

The antibodies to both Red 1 and Red 2 could detect Red 1, Red 2 and cyt P-450 reductase in microsomes. This indicates that the antibodies are identical. Anti-Red 2 also recognized the minor band of Red 1. The result suggests that the minor band in Red 1 has to be a degradation product as Red 2 was not contaminated when used for immunization purposes.

Immunochemical studies on the cyt P-450 reductase preparations of another organ of the same animal (ovine) was investigated to determine if the enzymes were related immunochemically. Immunoblotting studies on a partially purified ovine liver cyt P-450 reductase preparation showed

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that the liver enzyme could be detected by the antibodies against the adrenal enzyme. The ovine liver cyt P-450 reductase has the same Mr as the ovine adrenal enzyme, as judged by the position of the bands on the immunoblot. This result corresponds with the hypothesis suggested earlier that the cyt P-450 reductase from different organs within the same animal is coded by the same gene [3, 8, 13].

The investigation of the immunochemical relationship between the cyt P-450 reductase of two different species was undertaken. Bovine adrenal cyt P-450 reductase was detected by the antibodies against ovine adrenal cyt P-450 reductase, but to a much lesser extent. This indicates that there is a definite immunochemical relationship between the ovine adrenal and the bovine adrenal enzymes, which is not as strong as between the two ovine (liver and adrenal) enzymes. It is clear from the results obtained with immunoblotting studies that the antibodies prepared in this study are only suitable to detect ovine cyt P-450 reductases and not cyt P-450 reductases from other species. It was also found with immunoblotting that adrenocortical mitochondrial ADXR and adrenocortical microsomal cyt P-450 reductase are not immunochemically related.

Immunoassays are very specific and require relatively low concentrations of protein. This feature of immunoassays makes them ideal for the detection of small quantities of protein. The antibodies raised against ovine adrenal cyt P-450 reductase will in future be used in immunoassays to determine the levels of cyt P-450 reductase in ovine fetal development. The cloning of the ovine cyt P-450 reductase gene in cos-1 cells is a future prospect and the identification of the cloning product will be determined by immunological techniques.

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CHAPTER 7

EXPERIMENTAL

7.1. Preparation of microsomes

The liver and adrenal glands of freshly slaughtered sheep and cattle were removed and kept on ice to minimize protein denaturing and to inhibit protease activity.

7.1.1. Preparation of ovine adrenal microsomes

All glassware used for the preparation of microsomes were rinsed with sucrose solution (0.25 M, pH 7,4-7,8). All procedures were performed at 4°C and all buffers were pre-cooled to 4°C before use. Adrenal glands were frozen at -20°Cin aliquots of 70 g after removal of the fat. When used for microsome preparation the capsules were removed from the frozen glands which were cut into small pieces. The glands were homogenized in a Warring Blendor to a very fine suspension in sucrose solution (250 ml per 50 g adrenal glands). The suspension was subsequently homogenized at 4°C with a motor-driven homogenizer and the homogenate centrifuged at 860 x g for 10 minutes at 4°C to remove cell debris. The supernatant was centrifuged at 10 000 x g for 10 minutes and then at 12 500 x g for another 10 minutes to remove the mitochondria. The resulting supernatant was subsequently ultracentrifuged for 60 minutes at 70 000 x g and the pellet, which contained the microsomes, resuspended by homogenization in enough KCl-solution (0,15 M) to form a thin paste. The resuspended microsomal preparation was centrifuged again at 70 000 x g for 30 minutes. The resulting pellet was resuspended by homogenization in the smallest possible volume of Tris-sucrose-buffer (Tris 50 mM,

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sucrose 0.25 M, pH 7,4-7,8) and stored in 0,5 or 1 ml aliquots in Eppendorf-tubes in liquid nitrogen [100].

7.1.2. Preparation of bovine adrenal microsomes

The adrenal glands were frozen at -20°C in aliquots of 80 g after removal of the fat. The cortex was separated from the rest of the frozen adrenal gland and used for microsomal preparation. The same preparation procedure as described for ovine adrenal microsomes was used.

7.1.3. Preparation of ovine liver microsomes

An ovine liver was removed from the animal and perfused with ice cold saline-solution (NaCl 0,9% w/v) to remove blood. The liver tissue was cut into small pieces and stored in aliqouts of 70 g as described for ovine adrenal glands. For the preparation of microsomes the pieces of liver were cut into yet smaller pieces with a scalple and the microsomes prepared as described earlier for ovine adrenal microsomes.

7.2. Purification of cyt P-450 reductase from ovine liver and adrenals and bovine adrenals

All operations were performed at 4°C and all buffers were pre-cooled to 4°C before use.

7.2.1. Reductase Assay

A solution of cyt c (0,5 mM) and NADPH (0,04 M) was prepared in potassium phosphate buffer (150 mM, pH 7,7). The reductase solution was diluted to a final volume of 0.9 ml in potassium phosphate buffer (150 mM, pH 7,7). The solution

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was mixed with cyt c (0,5 mM, 200 µl) and used as a reference for the spectophotometer. Reduction was initiated with NADPH (0,04 M, 10 µl). The progress of the reaction was followed by monitoring the increase in absorbance at 550 nm for 2-3 minutes [101].

7.2.2. Procedure 1

This procedure is a slightly modified version of a procedure used by Estabrook [97] to purify rat liver cyt P-450 reductase.

Ovine adrenal microsomes (0,9-1,8 g protein) were diluted 5 fold with buffer 1 (Tris 27,5 mM, pH 7,7, ethylenediamine tetra-acetic acid (EDTA) 1,1 mM, glycerine 22%, deoxycholate 0,44% and Triton N101 0,88%), ultracentrifuged for 60 minutes at 100 000 x g and the supernatant poured through a cheese cloth.

Anion exchange

DE-52 (300-400 ml swollen gel) was packed in a Buchner funnel (5 cm x 8 cm) and equilibrated with 600 ml of buffer 2 (Tris 0,1 M, pH 7,7, Triton N101 0,5%, deoxycholate 0,1% and EDTA 1 mM). The supernatant was loaded onto the gel which was then washed, first with 1 l of buffer 1, followed by 1 l of buffer 1 containing 0,13 M KCl. The reductase was eluted by washing with 1 l of buffer 1 containing 0,3 M KCl.

Affinity chromotography

A 2'5'ADP-Sepharose column 4B (1,9 cm x 1,5 cm) was packed in buffer 1 and equilibrated with buffer 3 (potassium

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phosphate 10 mM, pH 7,7, glycerine 20%, Triton N101 0.1% and EDTA 1 mM). The eluate from the DE-52 column was loaded onto the 2'5'ADP-Sepharose column and washed with buffer 4 (potassium phosphate 150 mM, pH 7,7, glycerine 20%, Triton N101 0,1% and EDTA 1 mM). The enzyme was subsequently eluted with 1,2 mM $NADP^+$ dissolved in buffer 3. The yellow fraction was collected and concentrated by ultrafiltration to an approximate volume of 5 ml using an Amicon concentrator equipped with PM30 membrane filter (molecular mass cut off of 30 000). A few crystals $K_6Fe(CN)_6$ were added to the preparation to reoxidized the reductase.

Gel filtration

Sephadex G-200 was swollen in buffer 3 and equilibrated with the same buffer. The reoxidized enzyme was loaded onto the column (1,9 cm x 35 cm) and eluted with the same buffer. Two fractions were obtained. The first which contained the reductase, was concentrated to a final volume of 2-3 ml by ultrafiltration as described earlier and the second fraction contained the unused $K_6Fe(CN)_6$.

Anion exchange

A hydroxylapatite column (1,9 cm x 7 cm) was equilibrated with buffer 5 (potassium phosphate 20 mM, pH 7,7 and glycerine 20 %). After applying the sample, the column was washed with buffer 5 until the absorbance at 276 nm was less than 0,05. The enzyme was subsequently eluted with buffer 6 (potassium phosphate 150 mM, pH 7,7 and glycerine 20%). The solution containing the purified enzyme was dialyzed overnight against buffer 7 (potassium phosphate 100 mM, pH 7,7 and glycerine 20%) at 4°C and stored in vails at -70°C.

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7.2.3. Procedure 2

This procedure is the same as procedure 1 except that the hydroxylapatite column was replaced with a 40 ml DEAE-Sepharose CL 6B column (2,5 cm x 20 cm) equilibrated with buffer V (potassium phosphate 10 mM, pH 7,7). After loading the sample the column was washed with buffer VI (potassium phosphate 50 mM, pH 7,7) and the reductase eluted with buffer VII (potassium phosphate 50 mM, pH 7,7, KCL 0,5 M and deoxycholate 0,1 mM). The resulting enzyme was concentrated and dialyzed overnight against buffer VIII (potassium phosphate 50 mM , pH 7,7,) at 4^{o}C and stored at -20^{o}C [2].

7.2.4. Procedure 3

Ovine microsomes (1,8-2 g) were suspended in buffer A (potassium phosphate buffer 100 mM, pH 7,25, glycerine 20%, dithiotreitol (DTT) 1mM, EDTA 1 mM, Flavin adenine mononucleotide (FMN) 2 uM, buthylated hydroxytoluene (BHT) 23 uM and phenylmethansulfonylfluoride (PMSF) (0,4 mM) to a final protein concentration of 1,9 mg/ml. Dithiotreitol is a reducing agent, PMSF is a protease inhibitor and BHT is an antioxidant. Cholate (20%) was added to the preparation to yeald a final concentration of 0,7% (w/v). The preparation was stirred for 15 minutes on ice. After the preparation was centrifuged at 100 000 x g for 60 minutes it was diluted with buffer A to a final protein concentration of 1,5 mg/ml.

A 2'5'ADP-Sepharose 4B column (1,9 cm x 1 cm) was equilibrated with buffer B (potassium phosphate buffer 10 mM, pH 7,25, glycerine 20% and EDTA 1 mM). After the sample was loaded, the column was washed with buffer C (potassium phosphate 300 mM, pH 7,25, EDTA 0,1 mM, glycerine 20% and Triton N101 0,1%). The column was subsequently washed with buffer D (potassium phosphate 30 mM, pH 7,25,

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EDTA 1 mM, glycerine 20% and deoxycholate 0,15%) and the reductase eluted with buffer E (potassium phosphate 30 mM, pH 7,7, EDTA 1 mM, glycerine 20%, deoxycholate 0,15%, NADP 1,3 mM and PMSF 0,4 mM). Only fractions with an $A_{455}:A_{380}$ ratio of more than one were collected. The enzyme solution was dialyzed overnight against buffer F (potassium phosphate buffer 30 mM, pH 7,7, EDTA 0,1 mM and glycerine 20%) and stored at -20°C [99].

7.2.5. Procedure 4

This method was used to isolate cyt P-450 reductase from ovine adrenals, ovine liver and bovine adrenals respectively. Microsomes (1,8 g protein) were suspended in buffer i (Tris-HCl 25 mM, pH 7,7, Triton N101 or Emulgen 913 0,8%, deoxycholate 0,4%, glycerine 20% and EDTA 1 mM) to give a protein concentration of 10-13 mg/ml. The preparation was subsequently ultracentrifuged at 100 000 x g for 60 minutes at 4°C.

Anion exchange

DE-52 (300-400 ml swollen gel) was poured onto a Buchner funnel (5 cm x 8 cm) and equilibrated with buffer ii (Tris-HCl 25 mM, pH 7,7, deoxycholate 0,2% and EDTA 1 mM). The supernatant was loaded onto the gel and washed with 1 l of buffer ii, followed by 1 l of a KCl solution (0,12 M) in buffer ii. The enzyme was eluted with 0,3 M KCl in buffer ii as a yellow solution which was then diluted with an equal volume of 20% glycerine.

Affinity chromotography

A 2'5'ADP-Sepharose 4B column (1,9 cm x 1,5 cm) was equilibrated with buffer iii (potassium phosphate 10 mM, pH

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7,7, glycerine 20%, Triton N101 or Emulgen 913 0,1% and EDTA 1 mM). The enzyme solution was applied to the column and washed with buffer iv (potassium phosphate 150 mM, pH 7,7, Triton N101 or Emulgen 913 0,1%, EDTA 1 mM and glycerine 20%) and eluted with buffer v (1,2 mM NADP or 2,5 mM 2'AMP solubilized in buffer iii). When 2'AMP was used the pH of the buffer was adjusted to 7,4. The enzyme solution was concentrated by ultrafiltration as described earlier to a final volume of 3 ml and a few crystalls of $K_3Fe(CN_6)$ added to the solution to reoxidize enzyme.

Gel filtration

The sample was subsequently loaded onto a Sephadex G-200 column (1,9 cm x 35 cm) equilibrated with buffer iii and eluted with the same buffer. Two yellow coloured fractions appeared of which the first contained the reductase. This fraction was concentrated by ultrafiltration to a volume of 3-5 ml as described previously.

Anion exchange

A hydroxylapatite column (1,9 cm x 1,5 cm) was equilibrated with buffer vi (potassium phosphate 20 mM, pH 7,7, glycerine 20% and EDTA 1 mM). The sample was loaded onto the column and washed with the same buffer until the absorbance of the eluate at 280 nm was less than 0,02. The enzyme was eluted with buffer vii (potassium phosphate buffer 100 mM, pH 7,7 and glycerine 20%).

The enzyme was concentrated by ultrafiltration as described previously and dialyzed overnight against buffer viii (potassium phosphate buffer 100 mM, pH 7,7, glycerine 20% and EDTA 0,2 mM) and stored at -20^{o}C [98].

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7.3. Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

7.3.1. Preparation of Running gel (10%) and Stacking gel (3%)

The SDS-PAGE-cell was assembled and the lower part of the gel compartment sealed off with melted agarose solution (0,1%). Running gel buffer (Tris-HCl 0,375 M, pH 8,8 and SDS 0,1%), (10 ml) was mixed with running gel monomer stock (5 ml) (acrylamide 30%, N,N-bismethylacrylamide 0,8% solubilized in running gel buffer). Freshly prepared ammoniumperoxodisulphate solution (10%)(100 µl) was added to the running gel mixture. N, N, N, N-tetramethylethylenediamine (TEMED) (10 µl) was added to this mixture and directly thereafter the gel was poured. After the running gel had polimerized stacking gel buffer (Tris-HCl 0,125 M, pH 6,8 and SDS 0,1%)(4,5 ml) and stacking gel monomere (acrylamide 30%, N,N,-bismethylacrylamide 0,8% solubilized in stackinggel buffer)(0,5 ml) were mixed, freshly prepared ammoniumperoxodisulphate (50 µl) and TEMED(5 µl) added and the gel poured. A comb was inserted into the stackinggel to form wells for sample application.

7.3.2. Preparation of samples

Protein solutions (1-5 µg protein) were loaded onto the gel. All samples were diluted with an equal volume of treatment buffer (Tris-Cl buffer 67,5 mM, pH 6,8, SDS 2%, glycerine 10% and 2-mercaptoethanol 5%) and bromophenol blue-solution (1 µl) as a frontmarker and boiled for 1,5 minutes. The samples were allowed to cool to roomtemperature before loading it onto the gel.

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7.3.3. Electrophoresis

The upper and lower buffer chambers of the electrophoresis cell were filled with tankbuffer (Tris buffer 25 mM, pH 8,3, glycine 192 mM and SDS 0,1%). After loading of the proteins, a constant current of 20 mA was applied until the Bromophenol Blue reached the end of the running gel. The gel was subsequently removed to be stained or used in immunoblotting experiments.

7.3.4. Staining and Destaining procedures

The gel was stained for 1 hour in a Coomassie blue solution (Coomassie blue R-250 0,125%, methanol 50% and acetic acid 10%) The staining solution was removed and the gel destained first in solution 1 (methanol 50% and acetic acid 10%) for an hour and then in solution 2 (acetic acid 7% and methanol 5%) overnight.

7.4. Isoelectric focussing

7.4.1. Preparation and the electrophoresis

The plate of the isoelectric focussing apparatus was pre-cooled to 4^{o}C. Paraffin liquid was distributed evenly onto the plate as a heat exchange fluid. The Precoat (pH 3 to pH 10, 300 μm thick) was placed onto the cooling plate avoiding the trapping of bubbles and the cover sheet was removed from gel carefully. Two wicks were saturated with respectively 0,02 M acetic acid and 0,02 M sodiumhydroxide and the excess fluid was blotted. The wick saturated with acid was placed at the anode and the wick saturated with hydroxide was placed at the cathode. The electrodes were placed into position making complete contact over the whole wick area.

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An applicator strip was placed in the middle of the gel parallel with the electrodes and the samples (10 μg) were applied into the slots of the applicator strips. The proteins were separated with constant power for 2 to 3 hours.

7.4.2. Staining and destaining procedures

The gel was removed and the protein bands were fixed with a 10% trichloroacetic acid solution for 10 minutes. The gel was then rinsed with distilled water and stained with Coomassie blue stain used in SDS gel staining, for 15 minutes. The gel was destained first with destain solution 1 for 15 minutes and then with destain solution 2 for 15 minutes. The gel was allowed to airdry.

7.5. Optical absorption spectra

Optical absorption spectra were measured with a Beckman DU-65 spectophotometer at roomtemperature (25°C). Quarts mini cuvetts were used with a capacity of 1 ml. The spectra were measured over a wavelength range of 320 nm to 550nm.

7.6. Preparation of hydroxylapatite

$CaCl_2$ (0,5 M)(2 l) and Na_2HPO_4 (0,5 M)(2 l) were allowed to run at equal rates (12-15 ml/min) into a beaker while stirring continuously at a constant rate. The precipitate was allowed to settle, the supernatant sucked off with a vacume pump and the precipitate washed 4 times with water (3 l), allowing complete settling of the crystalls after each wash. The precipitate was diluted to 3 l with distilled water and freshly prepared NaOH solution (40%)(100 ml) added while stirring. The mixture was heated to 100°C within 45

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minutes and boiled gently for 60 minutes while stirring. The supernatant was removed after the precipitate was allowed to settle for 5 minutes. The precipitate was washed 4 times with water (3 l), allowing 5 minutes settling time between each wash. The precipitate was suspended in sodiumphosphate buffer (0,01 M , pH 6,8)(4 l) and heated to 100°C while stirring. This procedure was repeated twice with 0,01 M and twice with 0,001 M sodiumphosphate buffer (pH 6,8) allowing 15 minutes boiling each time. The final suspension was resuspended in the sodiumphosphate buffer (0,001 M) (107).

7.7. Purification of Cholate

Cholate was solubilized in ethanol (50%) in a sonic bath and the solution poured through a Buchner funnel containing equal quantities of activated charcoal and celite. The filtrate was dried under vacuum and resuspended in ethanol (50%). The process was repeated and the final crystalls are dried further in a vacume oven overnight [8].

7.8. Protein determinations

7.8.1. Bradford method

Coomassie brilliant blue G-250 (500 mg) was dissolved in ethanol (95%, 50 ml) and phosphoric acid (85%, 100 ml) added to the mixture . The resulting solution was diluted to a final volume of 1 l with distilled water and the solution filtered. The protein standard used was bovine serum albumin (BSA) with a concentration of 1 mg/ml in basal buffer (potassium phosphate 50 mM, pH 7,4, EDTA 0,1 mM, DTT 0,1 mM and glycerine 10%). Protein standards ranging from 0 μg-100 μg protein/100 μl with 20 μg intervals were prepared in basal buffer. Bradford-reagent (5 ml) was added to every

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tube and the absorbance at 595 nm determined immediately using the 0 μg protein standard solution as the reference. A standard curve was plotted from which unknowns could be determined.

7.8.2. Folin-Lowry method

A fresh alkaline solution was prepared on the day of use by mixing an alkaline sodium carbonate solution (2% $NaCO_3$ in 0,1 M NaOH, 50 ml) and a copper sulphate sodium potassium tartrate solution (0,5% $CuSO_4.5H_2O$ in 1% NaKtartrate, 1 ml). The commercial Folin Ciocalteau-solution was diluted with an equal volume of distilled water. Protein standards ranging from 1-100 μg/ml with 20 μg intervals were prepared using basal buffer as diluent and a BSA solution (1 mg/ml). Alkaline solution (5 ml) was added to each tube. The tubes were subsequently incubated at room temperature for 10 minutes, Folin Ciocalteau reagent (0,5 ml) was added to each tube and the resulting mixtures were incubated at room temperature for 30 minutes. The absorbance at 750 nm was determined using the 0 μg/ml solution as a reference. A standard curve was constructed to determine unknowns.

7.8.3. Pierce method

Protein standards ranging from 0 to 120 μg/ml with 20 μg intervals were prepared using a BSA solution (2 mg/ml) provided in the Pierce-kit (Pierce chemical company). The working reagent (1 part reagent B and 50 parts reagent A, 2 ml) was added to every tube which was incubated for 30 minutes at 37°C. The contents was allowed to cool to room temperature and the absorbance at 562 nm determined with water as a reference. A standard curve was plotted from which unknowns were determined.

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7.9. Preparation of antibodies against cyt P-450 reductase

7.9.1. Preparation of naked bacteria-protein-complexes

A suspension of naked bacteria (NB)(1 mg/ml) was prepared in a small homogenizer. Subsequently the NB suspension and solution of protein (cyt P-450 reductase) was mixed in a small pear-shaped flask, at a ratio of NB:protein (m/m) of 5:1. This mixture was dried on a rotary evaporator and the resulting precipitate resuspended by brief sonication in PBS to a final concentration of 240 μg of protein-NB complex/0,5 ml PBS. For the first two immunizations 1 ml of a 120 μg protein-NB-complex/0,5 ml suspension was prepared by dilution with PBS.

7.9.2. Immunization schedule

Rabbits were immunized with this suspension over a period of 39 days [13]. A day 0 control blood sample was drawn before the first injection. The first two injections given on day 1 and day 4, were 0,25 ml and 0,5 ml respectively of a 120 μg protein-NB complex/0,5 ml suspension. Further injections on days 7, 11, 18, 21, 25, 32, 35 and 39 were 0,5 ml of a 240 μg protein-NB-complex/0,5 ml suspension. On days 11, 18, 25, 32 and 35 a 2 ml volume of blood was drawn while 20 ml was drawn on day 46.

7.9.3. Antiserum preparation

The blood samples were allowed to clot by incubation at 37^{o}C for 30 minutes and the clot was subsequently removed by centrifugation in a bench top centrifuge at maximum speed (800 x g). The supernatant, i.e. serum, was stored at -20^{o}C in Eppendorf tubes.

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7.10. Enzyme Linked Immunosorbent Assay (ELISA) for cyt P-450 reductase antibodies.

The protein (cyt P-450 reductase) was diluted to 1 μg/ml with bicarbonate buffer ($NaHCO_3$, 50 mM, pH 9,6) and ELISA plates (Nunc maxisorp with certificate) were coated with this solution (100 μl/well overnight at 4°C in wells 1-11). The solution was decanted and non-specific binding was blocked with 0,1% casein in carbonate buffer (200 μl) in wells 1-12 for 1 hour at 37°C. The plates were then washed three times with PBS-Tween (PBS containing 0,1% Tween 20). This washing procedure was repeated after each of the subsequent incubation steps. A dilution series of antiserum (100 μl/well) was made in wells 2-11. Casein buffer and antiserum (diluted 1 in 20) were used as controles in wells 1 and 12 respectively. The plates were then incubated for 1 hour at 37°C. Sheep-anti-rabbit antiserum (diluted 1 in 400 in casein buffer) (100 μl/well) was pipetted into all wells and incubated at 37°C for 1 hour. Rabbit-peroxidase-anti-peroxidase (rabbit-PAP)(diluted 1 in 10 000 in casein buffer, 100 μl) was then pipetted into all wells and incubated at 37°C for 1 hour. The substrate was prepared just before use by dissolving 0,01 g 2,2'azino-di [3 ethylbenzthiazoline-sulfonic acid (6)] (ABTS) in citrate buffer 0,1 M, pH 5 and finally adding 10 μl H_2O_2. This solution (150 μl) was pipetted into all wells and the absorbance at 405 nm was recorded after 10 minutes.

7.11. Electroblotting

7.11.1. Electro-transfer

Proteins (cyt P-450-reductase) were separated on a 10% SDS-PAGE (as described previously in Section 7.3) and transfered to a nitrocellulose membrane as described by Strott [104].

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The nitrocellulose membrane was saturated with transfer buffer (Tris buffer 0,05 M, pH 8,3, glycine 0,25 M and methanol 20%) and placed on one side of the gel. Care was taken to ensure complete contact over the entire area of the gel. The position of the gel was marked on the nitrocellulose membrane with a ballpoint pen. The gel and membrane was sandwiched between scouring pads ("Scotch Brite") and blotting paper, saturated with transfer buffer. A perspex grid was placed on both sides of the sandwich which was then inserted into a tank of transfer buffer with the nitrocellulose membrane facing the anode and the SDS-gel facing the cathode. A constant current of 40 mA was applied across the gel overnight.

7.11.2. Immunoblotting

Following transfer from the SDS-PAGE gel to the nitrocellulose membrane, the membrane was blotted dry with blotting paper and the non-specific binding on the membrane blocked by incubation for 20 minutes with casein buffer (NaCl 154 mM , casein 0,5%, Tris-HCl 10 mM, thiomersal 0,02%, all reagents are dissolved and the pH adjusted to 7,6 before casein was added and stirred slowly overnight at 4^{o}C). The membrane was subsequently incubated for 2 hours with an optimal dilution of the rabbit antiserum against cyt P-450 reductase. (All antisera were diluted in casein buffer.) The membrane was washed 4 times for 4 minutes with PBS-Tween. Subsequently a 1 hour incubation period with sheep-anti-rabbit antibodies (1 in 200 dilution) was followed by washing with PBS-Tween as described before. The membrane was then incubated for 1 hour with rabbit-PAP (1 in 5000 dilution), followed by washing with PBS-Tween. The membrane was finally incubated (usually 5 to 10 minutes) with the substrate until blue coloured bands appeared on the

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nitrocellulose membrane. The membrane was washed with distilled water, blotted dry and stored in aluminium foil.

The substrate solution was prepared just before use by dissolving 30 mg 4-chloro-naphtol in 15 ml 95% methanol and then mixed with 50 ml PBS and 15 μl H_2O_2.

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