An investigation into the complex formation of membrane bound cytochrome b5 isolated from ovine liver microsomes

by

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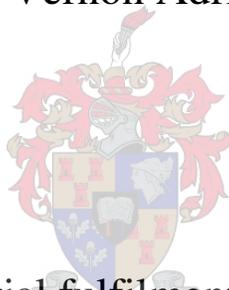
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DECLARATION

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

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Summary

Membrane bound cytochrome b5 is a ubiquitous protein with an average molecular weight of 16 kDa. The protein is involved in a number of reactions providing electrons directly to cytochrome P450 enzymes or to other enzymes involved in lipid biosynthesis. It is also known that the protein influences the activities of certain enzymes via an allosteric effect. It has been accepted in the literature that the cytochrome b5 exists primarily in the monomeric form, however, recently it has been shown that it forms homomeric complexes *in vivo*. In this study, we investigate the cytochrome b5 complex formation using a variety of analytical tools. Cytochrome b5 was isolated from ovine liver microsomes and the purity verified using sodium dodecyl sulphate polyacrylamide gel electrophoresis and electrospray ionisation mass spectrometry. The latter analysis confirmed the presence of a single heme containing protein with M_r=15865 Da, while separation on the polyacrylamide gel revealed oligomeric complex formation with the tetrameric form the most prominent oligomer. Using different and particularly harsh denaturing conditions we found that the observed oligomeric aggregates persisted, indicating highly stable complexes. The most prominent tetrameric aggregate was identified to be cytochrome b5 by mass spectrometric sequencing. Further complex formation studies, using a fluorescent dye (1-anilinonaphthalene-8-sulfonic acid) that interact with hydrophobic cavities formed during oligomerisation, provided evidence of protein assembly in oligomeric complexes or aggregation. The formation of the cytochrome b5 complexes was dependent on ionic strength and protein concentration. Previously it was shown that the hydrophobic membrane anchoring domain plays a pivotal role in the cytochrome b5's homomeric complexes. Using a peptide (IITTIDSNSS), resembling a portion of this domain, together with circular dichroism we showed more organized structure present for the wild-type peptide vs. a mutated control peptide (LLSSLKAVAV). A modified ELISA interaction assay also revealed that the wild-type peptide had a specific interaction with cytochrome b5, providing further evidence that the membrane anchoring domain plays a role in complex formation. These studies also indicated that a hydrogen bond network in this domain may be important for the formation of the homomeric complexes of cytochrome b5.

Opsomming

Membraan-gebonde sitochroom b5 is 'n alomteenwoordige proteïen met 'n gemiddelde molekulêre massa van 16 kDa. Die proteïen is betrokke in reaksies waar dit elektrone direk aan sitochroom P450 ensieme verskaf, sowel as ensieme betrokke in lipiedbiosintese. Dit is ook bekend dat die proteïen die aktiwiteite van sekere ensieme via 'n allosteriese effek beïnvloed. Dit is geredelik in die literatuur aanvaar dat sitochroom b5 as 'n monomeer voorkom, maar daar is kort gelede gerapporteer dat homomeriese komplekse *in vivo* vorm. In hierdie studie is die sitochroom b5-kompleksvorming ondersoek deur gebruik te maak van verskeie analietiese metodes. Sitochroom b5 is vanuit skaaplewer mikrosome geïsoleer en die suiwerheid met behulp van natrium-dodesiel-sulfaat-poliakrielamied-gel-elektroforese en elektrosproei-ionisasie massa-spektrometrie geverifieer. Met die laasgenoemde bevestig dat 'n enkele heem-bevattende proteïen met M_r =15865 teenwoordig was, terwyl poliakrielamied gel-skeiding kompleksvorming getoon het, met tetrameer as die mees prominente oligomeer. Deur verskeie denaturerings kondisies, intsluitend besondere kondisies, is gevind dat hierdie aggregate behoue bly, wat baie stabiele oligomere aandui. Die mees prominente tetrameriese aggregaat is as sitochroom b5 geïdentifiseer met behulp van massa spektrometriese volgordebepaling. Kompleksvorming is verder bewys deur 'n verdere ondersoek met behulp van 'n fluoresserende kleurstof (1-anilinonaftaleen-8-sulfoonsuur) wat met die hirdofobiese holtes, wat vorm tydens oligomermerisasie, interaksie het. Die kompleksvorming was afhanklik van ioniese sterkte, sowel as proteïenkonsentrasie. Voorheen was dit bewys dat die deurslaggewende faktor in die vorming sitochroom b5 se homomeriese komplekse die hidrofobiese membraan-anker-domein is. Deur gebruik te maak van 'n peptied (IITTIDSNSS) wat lyk soos 'n gedeelte van hierdie domein, tesame met sirkulêre dichroisme, is gewys dat meer georganiseerde struktuur teenwoordig was vir die wilde tipe peptied vs. 'n gemuteerde kontrole peptied (LLSSLKAVAV). 'n Gemodifiseerde ELISA-interaksie-essai het ook getoon dat die wilde-tipe peptied spesifieke interaksie met sitochroom b5 het, 'n verdere bewys dat hierdie membraan-anker-domein 'n rol speel in kompleksvorming. Hierdie studies het ook aangedui dat 'n waterstofbinding netwerk in die domein belangrik kan wees vir die vorming van die homomeriese komplekse van sitochroom b5.

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Figure 5.1: Schematic representation of the mELISA for cytochrome b5-peptide interaction. The assay involves the immobilisation of cytochrome b5, followed by the incubation with a biotinylated-peptide where after the HRP-avidin conjugate is applied. An appropriate substrate is added for colorimetric visualisation (Biotin and streptavidin has one of the strongest non covalent interactions known in nature. The complex has a calculated dissociation constant (K_d) of 10^{-14} mol/L.

Figure 5.2: Circular dichroism spectra for various protein secondary structures. Spectra represents: solid line, α-helix; long dashed line, anti-parallel β-sheet; dotted line, type 1 β-turn; crossed dashed line, extended 3_{10}-helix or poly (Pro) II helix and short dashed line, irregular structure.

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Figure 5.7: Affinity of biotinylated-peptides for immobilised cytochrome b5. Biotinylated-peptides (2.7 µg) were incubated with 500 ng immobilised cytochrome b5 where after streptavidin-HRP conjugate (1: 5000) was added. Wt-Peptide; wild-type peptide, C-Peptide; control peptide, No Peptide; No peptide added to 500 ng cytochrome b5 and Buffer; no cytochrome b5 immobilised.

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Figure 5.9: Saturation binding curves of biotinylated-peptides. Biotinylated-peptides (2.7 µg) was added to increasing amounts of immobilised protein. The presence of the biotinylated-peptide was determined using streptavidin-HRP conjugate (1: 5000) in duplicate analyses.

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List of abbreviations

17α-OH-PREG	17-hydroxy-pregnenolone
17α-OH-PROG	17-hydroxy-progesterone
3βHSD	3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase
ANS	1-Anilinonaphthalene-8-Sulfonic Acid
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CaCl$_2$	Calcium Chloride
CD	Circular Dichroism
c-DNA	*Chromosomal* Deoxyribonucleic Acid
CPR	NADPH-Cytochrome P450 reductase
DEAE-cellulose	Diethylaminoethyl Cellulose
DHEA	Dehydroepiandrosterone
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbant Assay
ER	Endoplasmic Reticulum
ESI-MS	Electrospray Ionisation Mass Spectrometry
FRET	Fluorescence Resonance Energy Transfer
HPGPC	High Performance Gel Permeation Chromatography
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidise
ID	Inner Diameter
kb	Kilobase
KCl	Potassium Chloride
kDa	Kilodalton
KOH	Potassium Hydroxide
mELISA	Modified Enzyme Linked Immunosorbant Assay
MS	Mass Spectrometry
mV	Millivolts
NaCl	Sodium Chloride
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NaSCN	Sodium Thiocyanate
nLC - MS/MS	nano Liquid Chromatography Mass Spectrometry/Mass Spectrometry
P450c17	Cytochrome P450c17
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
SDS	Sodium Dodecyl Sulphate
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis
TEMED	*N,N,N,N*-tetramethylenediamine

TRIS-acetate	Tris(hydroxymethyl)aminomethane Acetate
TRIS-HCl	Tris(hydroxymethyl)aminomethane Hydrochloride
UP-LC	Ultra Performance Liquid Chromatography
UV	Ultra Violet
UV-VIS-spectroscopy	Visible and Ultraviolet Spectroscopy

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Chapter 1

Prelude

Cytochrome b5 is a small acidic protein which serves as an electron carrier for several biochemical reactions [1]. Two forms of cytochrome b5 exist in mammals, namely a smaller hydrophilic isoform (14.6 kDa) [2] mainly found in the erythrocytes, and a membrane bound form (15.2 kDa) [3], amphiphilic in nature containing both a hydrophilic and hydrophobic region, the latter being responsible for targeting the protein to the fluid membrane [1].

The heme containing cytochrome b5 serves as an electron carrier for several monooxygenase reactions [1]. During this catalysis the hemoprotein is believed to provide the second, rate limiting electron to a number of cytochrome P450-dependent enzymes. Furthermore, the protein also supplies electrons to certain desaturases in the production of unsaturated fatty acids during lipid biosynthesis [1].

The isolation of the cytochrome b5 has been previously reported in literature [4] and the molecular mass has been reported to be an average molecular weight of 15.263 kDa [3]. Lombard *et al*. [3] accurately determined the molecular weight of ovine cytochrome b5, using electrospray ionisation – mass spectrometry (ESI-MS), and indicated a molecular weight of 15.263 Da. Furthermore, they reported that when isolating the protein from animal tissue a number of bands identified as protein multimers could be visualised using SDS gel electrophoresis (SDS-PAGE) [5]. When removing the membrane anchoring domain by trypsin digestion, these multimeric complexes were not observed. It was then concluded that the membrane anchoring domain plays an important role in the aggregation of the protein [6].

In this study, the complex formation of cytochrome b5 was further investigated. The goals of the study were the following:

1. the isolation and characterization of ovine liver cytochrome b5,
2. investigation of the aggregate formation, and
3. investigating the region suspected to be responsible for aggregation using synthetic peptides.

This study to reach each of the goals is reported in five chapters as outlined below.

Chapter 2 – Literature review

In Chapter 2 of this thesis, a literature review is given on cytochrome b5. The structure of cytochrome b5 is discussed with special emphasis on regions important for activity and aggregation, including the hydrophilic N-terminus, the heme binding pocket and the orientation of the hydrophobic C-terminus and the linker domain between the hydrophilic and hydrophobic domains. In addition, the physical and the chemical properties are discussed in detail. Cytochrome b5 influences a number of cytochrome P450-dependent reactions and there are a number of mechanisms postulated on this cytochrome b5–cytochrome P450-interaction. Chapter 2 also covers the four major hypotheses on cytochrome b5-cytochrome P450-interaction and discusses how cytochrome b5 influences specific cytochrome P450-dependent enzymes as well as enzymes involved in lipid biosynthesis.

Chapter 3 - Isolation and characterisation of cytochrome b5 from ovine liver

To investigate the aggregate formation and the region suspected for this phenomenon, full length cytochrome b5 had to be purified from ovine liver. In Chapter 3 the isolation of full-length cytochrome b5 from ovine liver microsomes is described. The isolation was accomplished by solubilising the microsomal membranes, thereby releasing the membrane bound proteins. The subsequent purification of cytochrome b5 was achieved using ion-exchange chromatography. The purified fraction was subjected to extensive dialyses and analysed using SDS-PAGE. Previous studies have reported the accurate mass of cytochrome b5 [3] and in this study the isolated protein was again subjected to high resolution electrospray mass spectrometry for accurate determination of its molecular weight. In order to achieve this, the amount of detergent left in solution was determined spectrophotometrically and trace amounts removed using commercially available spin columns. After solution clean-up, the accurate mass was determined which included the detection of its protoporphyrin IX heme group. The molecular mass detected correlated well with data previously reported in the literature [3].

Chapter 4 - Aggregation studies on cytochrome b5

Previous studies on cytochrome b5 showed that multimeric complexes are observed with SDS-PAGE. Chapter 4 describes the study in which these multimeric complexes were analysed using affinity purified immunoglobulins. The immunoglobulins recognising the

aggregated and monomeric forms of cytochrome b5 were isolated, by an immuno-affinty separation, and used in Western blot experiments. The various multimeric species of the purified protein were further analysed by SDS-PAGE and *nano* liquid chromatography mass spectrometry/mass spectrometry (nLC-MS/MS) using a peptide database.

In addition cytochrome b5 aggregation was also investigated using a fluorescent dye. The dye molecules accumulate in hydrophobic pockets resulting in an increase in the fluorescence.

Chapter 5 – Investigating the region responsible for aggregation using a synthetic peptide

Since it was demonstrated that the membrane anchoring domain plays a pivotal role in aggregate formation, it was decided to investigate this region using synthetic peptides. In Chapter 5, the aggregate formation was studied using circular dichroism and looking at differences in secondary structure of the commercially bought peptides. We investigated the effect of ionic strengths as well as different peptide concentrations. In addition to circular dichroism, the peptides were conjugated to biotin and this, in turn, was used for studying the interaction of immobilised cytochrome b5 with the biotinylated-peptides. For this, a technique commonly known as the ELISA was used whereby we immobilised purified cytochrome b5, added the biotinylated peptides and visualised the interaction spectrophotometrically.

Chapter 6 - Final thoughts

In Chapter 6 the findings of this study, based on the experimental data gathered, are discussed and future work is proposed.

Chapter 2

2. Literature Review on Cytochrome b5

2.1 Introduction

Cytochrome b5 is a ≈16 kDa heme containing protein [7] capable of taking part in redox reactions by accepting and transferring a single electron [8]. The hemoprotein exists as two forms: a cytosolic soluble form, functioning in the maintenance of the oxygen-carrying capacity of haemoglobin, and a membrane bound form, affecting cytochrome P450 reactions, either through direct electron transfers [9] or as an effector [10].

Cytochrome b5 was first observed more than five decades ago in microsomes and initially known as cytochrome *m* for their presence in microsomes [11]. It was found that this protein yielded an oxidised spectrum with a single absorbance in the Soret region at 414 nm. In the presence of a reducing agent, such as sodium dithionite, three major absorbance peaks could be observed, between 520 nm and 560 nm as well as at 424 nm. Unlike cytochrome P450 these reduced spectra were unaffected by carbon monoxide [11].

A number of forms of cytochrome b5 have been reported. These include forms with an average molecular weight of about 11 kDa [12], which were isolated through tryptic digestion. Another form of cytochrome b5, is the full-length form which was isolated from microsomes and which included reported molecular weights ranging 16 – 25 kDa [13]. Cytochrome b5 was isolated using detergents to solubilise membranes, releasing all membrane bound proteins. Recently, the accurate mass of cytochrome b5 was determined using ESI-MS and reported to be 15.263 kDa; excluding the protoporphyrin IX heme group [3], [6].

 It has long been reported that cytochrome b5 influences the activity of cytochrome P450-dependent reactions. These enzymes are largely responsible for xenobiotic and drug metabolism. During catalysis, cytochrome P450 needs two electrons and two protons for complete activity. The first electron is provided by its redox partner, NADPH-cytochrome P450 reductase (CPR). This, in turn, raises the redox potential allowing the second electron source to either be CPR or cytochrome b5. Cytochrome b5 can also alter the activity of the

enzymes by playing a role as effector to cytochrome P450. It has been shown that cytochrome b5 may also have no effect on the activity of certain P450 enzymes [1].

2.2 Structure of cytochrome b5

2.2.1 The cytochrome b5 gene

Two forms of cytochrome b5 exist, namely a soluble form and membrane associated form. The soluble cytochrome b5 is commonly found in erythrocytes and the membrane bound form is found embedded in the endoplasmic reticulum. The latter is known to be the full-length form of the cytochrome b5 that is 133 amino acids in length, while the soluble form consists only of approximately 93 amino acids. The membrane bound form of the protein is depicted in figure 2.1.

Using the complete nucleotide sequence of soluble cytochrome b5 chromosomal-DNA (*c*DNA) and a full-length protein *c*DNA clone for comparison, Zhang *et al.* [14] concluded that both the forms of the protein were transcribed from the same gene. The bovine cytochrome b5 gene was first isolated and characterised in 1993 by Christiano *et al.* [15]. They found that the gene had a span of about 28 kb and contained six exons which included a non-functional exon at the fourth position. Moreover, the size and organization of the genes were found to be comparable to that of the human and rabbit cytochrome b5 genes. The gene was found to follow the GT-AG rule, which is the observation that all introns in DNA begin with the nucleotides GT (guanine and thymine) and end with AG (adenine and guanine). Also, a number of G:C-rich Spl motifs were observed. This observation was indicative of a housekeeping gene – a constitutive gene required for basic cellular function. All of these were observed on the 5' flanking region further containing two CAAT boxes and lacking any TATA boxes [15]. The same was observed in the human gene [16].

Both Zhang *et al.* [14] and Christiano *et al.* [15] came to the same conclusion that only full-length cytochrome b5 is expressed and that the soluble form is a result of post transcriptional modification. A protease, such as trypsin, cleaves the membrane binding domain releasing the soluble, functional globular domain.
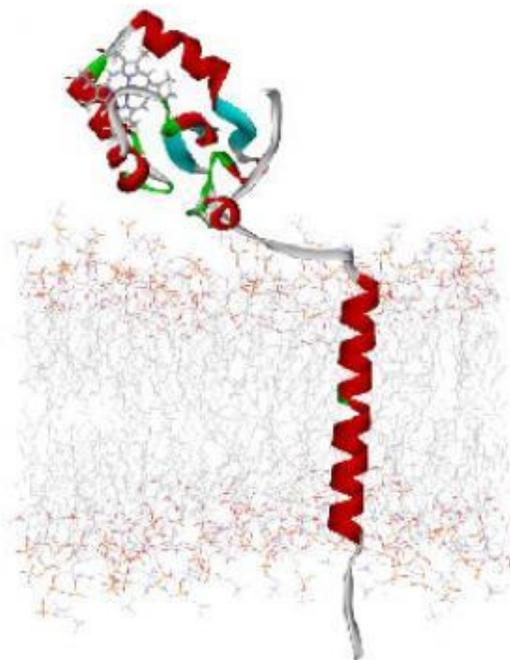
Figure 2.1: Molecular model of the membrane bound form of cytochrome b5. Full length cytochrome b5 with the C-terminus spanning the fluid membrane [17].

2.2.2 The hydrophilic N-terminus of cytochrome b5

As mentioned, full-length cytochrome b5 is about 133 amino acids in length. The protein is susceptible to proteases such as trypsin and chymotrypsin. Once treated with proteases a shortened, intact functional hydrophilic domain of cytochrome.b5 is produced.

The three dimensional structure of this fragment was determined by Mathews *et al.* [18] who found that the small acidic protein was cylindrical in shape. The dimensions of this "*head*" domain were 35 Ǻ in height and 30 Ǻ in diameter. Furthermore, it was found that this domain contained six relatively short helices and a single β-sheet strand; the latter dividing the hydrophilic domain in two. The larger of the two domains contains four helices which consist of residues 21 through to 78. The larger domain also houses the heme group allowing the domain to participate in electron transfer reactions [18].

2.2.3 Coordination of the heme-group

The soluble N-terminus of cytochrome b5 contains a hydrophobic crevice which houses the porphyrin ring and iron group (heme group). It is buried within the protein with the propionic acid groups facing the surface of the protein. Only part of these propionate groups are in contact with solvent. There are 14 side chains in contact with the heme group, with almost

80% of it being hydrophobic [18]. In animals, this heme is coordinated by two highly conserved histidine residues situated at positions 44 and 68. These histidine residues are aligned in an axial position to the heme group, with both heme-linked histidine side chains perpendicular to the porphyrin ring. The δ-nitrogens, which are hydrogen bound, form hydrogen bonds with main chain carbonyl oxygens [18]. The two ε-nitrogens of these residues are approximately 2.0 Ǻ from the iron group [19] forming a dative covalent bond between the iron and the nitrogen. This observation is in contrast to data published by Nunez *et al.* [20] who found in their tertiary structure calculations that the heme is covalently bound. It was found that there are no significant changes in the protein structure between oxidised cytochrome b5 and sodium dithionite reduced cytochrome b5. Some differences which were observed were that, in the presence of the reducing agent, it introduced a cation at the mouth of the hydrophobic pocket. Furthermore, to accommodate the binding of the cation, a slight movement of an adjacent lysine side chain was also observed. Argos and Mathews [19] went on to explain the significance of this cation. They concluded the reason for oxidised cytochrome b5 binding more strongly to cytochrome b5 reductase than reduced cytochrome b5, could be the fact that the cation, on the reduced cytochrome b5, could interfere with ionic attraction resulting in a decrease in charge pairing.

2.2.4 Structure of the linker

The linker domain is a short peptide, consisting of 14 amino acids, linking the membrane binding domain to the hydrophilic heme-containing domain. Little is known about the structure of the linker domain, which was first reported by Muskett *et al.* [21]. In a more recent study of the structure of cytochrome b5 [20] it was reported, using a construct consisting only of the globular head and the linker domain, that the linker domain had no secondary structure. Nunez *et al.* [20] also stated that the lack of structure observed in their studies could be due the fact that they were using a shortened version of the protein, rather than the full-length. Muskett *et al.* [21] found, using full-length cytochrome b5, that the linker was in an extended unfolded conformation. There is consequently still an uncertainty on the conformation and structure of the linker domain.

2.3 Distribution of cytochrome b5

Cytochrome b5 is a hemoprotein which is most abundant in the liver. Ichikawa and Yamano [22] reported cytochrome b5 to be found in the Golgi membranes of mammalian livers. They found that the cytochrome b5 content was relatively high, while other enzymes (cytochromes

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P450) are low. Also, it was found that the cytochrome b5 from these Golgi membranes yields a similar absorption spectrum as cytochrome b5 found in microsomes [22].

Apart from the liver, relatively high expression of cytochrome b5 is observed in the adrenal cortex. The adrenal cortex has been recognised to be responsible for the output of androgenic steroids [23]. The adrenal cortex is organised into three zones: an outer zone which is referred to as the zona glomerulosa, the middle zone known as the zona fasiculata and the most inner zone called the zona reticularis [24]. Mapes *et al.* [25] are believed to be the first to report the regional expression of cytochrome b5 in adrenals. They observed that cytochrome b5 was expressed only in the zona reticularis and was expressed together with cytochrome P450c17 (P450c17) and cytochrome P450 reductase and low levels of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3βHSD). In contrast to their findings, immunohistochemical studies by Engelbrecht [26] showed otherwise. Using a polyclonal antibody, raised against cytochrome b5 purified from ovine liver, cytochrome b5 was mainly detected in the zona fasiculata. Furthermore, cytochrome b5 was also unexpectedly detected in a cross section of the adrenal medulla. Engelbrecht [26], however, stated that latter finding could also be due to cross reactivity of the polyclonal antibody to a contaminating protein in the antigen preparation used to raise the antibodies.

2.4 Properties of cytochrome b5

2.4.1 Spectral properties of cytochrome b5

Like any other protein cytochrome b5 absorbs in the ultraviolet region. In addition, the protein also has an absorbance in the Soret region of the spectrum. Oxidised cytochrome b5 exerts an absorbance maximum in this visible region between 409 nm and 413 nm. Cytochrome b5 in a microsomal suspension can be rapidly reduced through the addition of a reducing agent, such as NADH. Upon reduction of cytochrome b5, the absorbance maximum is shifted from 413 nm to 426 nm. This shift is accompanied by the appearance of a further two peaks at 556 nm and 575 nm [27].

Spectral properties of cytochrome b5 are not only a useful tool for identification of the protein but can also be used to determine the molar concentration of the protein in a solution. The cytochrome b5 concentration can be determined using the change in absorbance at 426 and 409 nm together with an extinction coefficient of 185 mM^{-1}cm^{-1}. Alternatively, the appearance of the additional two peaks can also be used for the same purpose. This is done

by using the difference between the absorbance at 556 -and 575 nm and an extinction coefficient of 21 000 $M^{-1}cm^{-1}$. The same principle can be applied for the determination of cytochrome b5 content within a purified fraction. This is accomplished through the addition of sodium dithionite as reducing agent as it can reduce cytochrome b5 directly, in the absence of cytochrome b5 reductase [27].

2.4.2 Stability of cytochrome b5

The isolation of the membrane bound form of cytochrome b5 has been reported by Yang and Cederbaum [4]. This involves the preparation of microsomes through dehydration, which results in the formation of membranous vesicles. The stability of cytochrome b5 in a microsomal preparation has been investigated. The effects of freezing and storage of these preparations was reported by Pearce *et al.* [28], who found that longterm storage of microsomes had no significant influence on the activity of cytochrome b5. This long term storage included flash freezing, using liquid nitrogen, and storage for up to 6 months at −80°C. Also, long term storage of up to 2 years of cytochrome b5 in 250 mM sucrose at −80°C did not affect the concentration of protein when determined spectrophotometrically, concluding that cytochrome b5 within microsomes are relatively stable over long periods of storage.

The thermal stability of cytochrome b5 was investigated by Newbold and Whitford [29]. They reported that the full-length cytochrome b5 exhibited two transition states between a folded and an unfolded state over a temperature range of 5 – 95°C. These transitions were detectable at 43.4 and 71°C. Conversely, only a single transition was observed at 73°C for the truncated form of the protein. It was also reported that the addition of a mild detergent, such as Triton X-100, considerably lowered the melting point of the full-length version. What was also of note was that, as the concentration of the Triton X-100 was increased, the melting point decreased. Another effect of the detergent was the induction of a single, smoother transition between 40°C and 50°C [29].

Mitochondrial membrane cytochrome b5 are, amongst the different forms, the most thermal stable isoform of the protein known. Conversely, during the studies of Wang *et al.* [30]*,* only a single transition state could be observed for mitochondrial membrane and microsomal cytochrome b5. These states were observed at higher temperatures well above 70°C. Furthermore, they reported that the thermal stability could be advanced by swapping an invariant motif in the heme-independent folding core with a motif of that of a less stable

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microsomal cytochrome b5 isoform. This involved the substitution of Arg^{15} and Glu^{20} with histidine and serine respectively [30].

The effect of ionic strength on the stability of cytochrome b5 was reported by Schenkman [31]. Ionic strength plays a role in the interactions of cytochromes P450 with cytochrome b5, albeit to a lesser extent. It was shown that at higher ionic strength the interaction between cytochrome b5 and cytochrome P450 was diminished. Also, at low ionic strength the rate constant for cytochrome b5 reduction was increased 15-fold.

2.4.3 Membrane topology of cytochrome b5

Cytochrome b5 is embedded into the membrane of the endoplasmic reticulum (ER) via the C-terminus, unlike its redox partner and all other cytochromes P450 which are anchored via the N-terminus. The protein is synthesised on cytosolic ribosomes and was found to be inserted, post-transcriptionally, into the microsomal membranes. The insertion happens independently, without the use of any signal recognition particle [32]. This was observed when adding cytochrome b5 to microsomes which bound to the membrane in the presence of sodium carbonate, pH 11.5 containing 500 mM NaCl.

To determine whether the C-terminus spanned the lipid bilayer, or was located in the cytosol, Ozols [33] treated microsomes with trypsin and isolated the resulting hydrophobic peptides using gel filtration and HPLC to investigate this phenomenon. The amino acid sequence of cytochrome b5 contains a proline hinge region which is susceptible to the protease, trypsin [18]. Their findings suggested that cytochrome b5 is inserted into the lipid bilayer and that the hydrophobic domain contains a "hair-pin loop" resulting in the C-terminus to be in the same environment as that of the catalytic domain – the cytosol [33].

In contrast to these findings, Vergères *et al.* [34] reported that the membrane binding domain of cytochrome b5 spans the microsomal membrane and that the C-terminus is located in the lumen and not the cytosol. In their studies it was suggested that if the membrane binding domain spans the membrane, only the Lys^{93} (located in the cytosol) would be exposed for trypsin digestion and the other site, Arg^{127}, would be protected within the lumen. If this was the case, trypsin treatment would result in the single peptide, Pro^{94} – Asp^{133}, which would have a calculated molecular mass of 4.6 kDa. Otherwise, if both trypsin cleavage sites were exposed and accessible to trypsin, suggesting the hair-pin loop, it would result in a peptide 10 amino acids shorter and about 3.8 kDa in size. SDS-PAGE revealed that only the 4.6 kDa

peptide could be detected when cytochrome b5 was added to the microsomal membrane, but a shorter peptide could also be detected when digesting cytochrome b5 in the absence of membrane. Furthermore, carboxypeptidase Y had access to the C-terminus when cytochrome b5 was in solution but not when inserted into the membrane. These findings suggested that cytochrome b5 is inserted into the membrane with its C-terminus located in the lumen and not in a hair-pin loop configuration as suggested by Ozols [33] [34].

2.5 Mechanism of action

Cytochrome b5 assists in the catalyses of a number of reactions in steroidogenesis and the metabolism of xenobiotics within the liver. It has been shown that cytochrome b5 does this either through a direct electron transfer reaction (supplying a single electron to cytochrome P450 enzymes) or through an allosteric effect [1]. The manner in which cytochrome b5 enhances P450 enzyme activities is very important and little is known about its mechanism of action. A few mechanisms that have previously been postulated, discussed by Schenkman *et al.* [1] are summarised below.

2.5.1 Faster input of the second, rate limiting electron

All cytochrome P450 monooxygenase reactions require the input of two electrons during the conversion of substrate to product, via the insertion of a single molecular oxygen into the substrate. Cytochrome P450 enzymes are limited in that they can only accept a single electron at a time [35]. This electron is provided by NADPH via its redox partner, cytochrome P450 reductase. CPR reduces ferric cytochrome P450 to its ferrous state. The ferro-cytochrome P450 complexes with oxygen, forming an unstable oxy-ferro cytochrome P450 complex. This complex can readily dissociate resulting in the release of superoxide species which, subsequently, can further react forming hydrogen peroxide if a second electron is not introduced into the system. After the reduction of cytochrome P450 and complexation with oxygen, the complex midpoint potential for reduction is raised from a reported −230 mV to +50 mV [36]. With a midpoint potential of +5 mV, this makes cytochrome b5 a perfect candidate to provide the second rate limiting electron for the cytochrome P450 catalytic cycle to be completed. Figure 2.2 depicts this postulated mechanism of action [1].

11
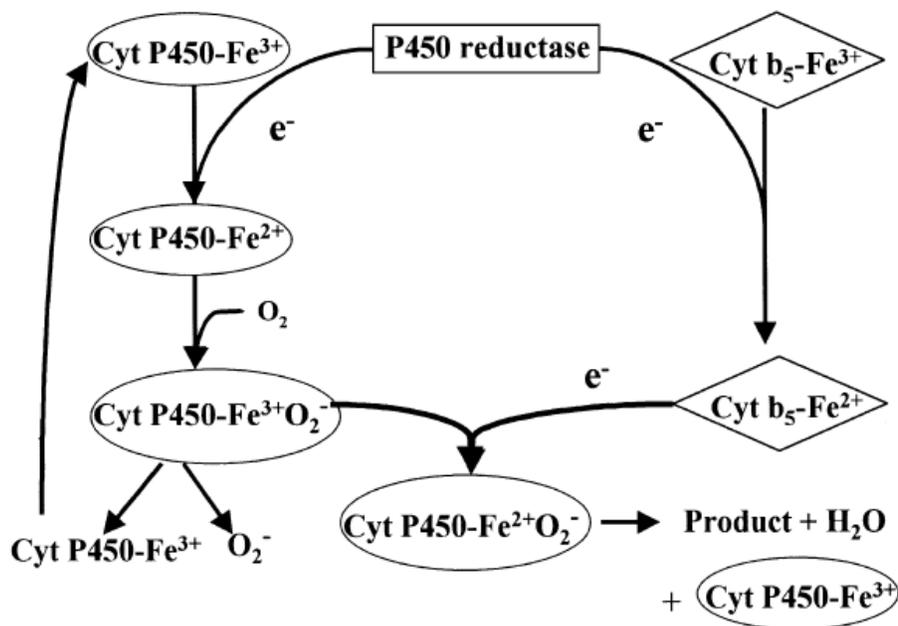
Figure 2.2: Supply of the second, rate limiting electron by cytochrome b5. Cytochrome b5 provides the rate limiting electron which promotes product formation rather than the dissociation of the oxy-ferro cytochrome P450 complex (leading to H_2O_2 formation) [1].

2.5.2 Prevention of uncoupling between cytochrome P450 enzymes and substrates

As discussed earlier, the oxy-ferro cytochrome P450 complex is very unstable and, when not reduced in time, results in the formation of superoxides; leading to the increase of hydrogen peroxide. Imai *et al.* [37] showed that the presence of cytochrome b5 improves NADPH oxidation through substrate oxidation. Gorskys *et al.* [38] demonstrated, by adding cytochrome b5, that they could increase the rate of product formation without the release of superoxides, thus decreasing the formation of hydrogen peroxide (figure 2.3). It was suggested by the authors that cytochrome b5 plays a vital role in providing the second electron faster than the release of the superoxides (uncoupling) thereby allowing the formation of more product [38] .

12
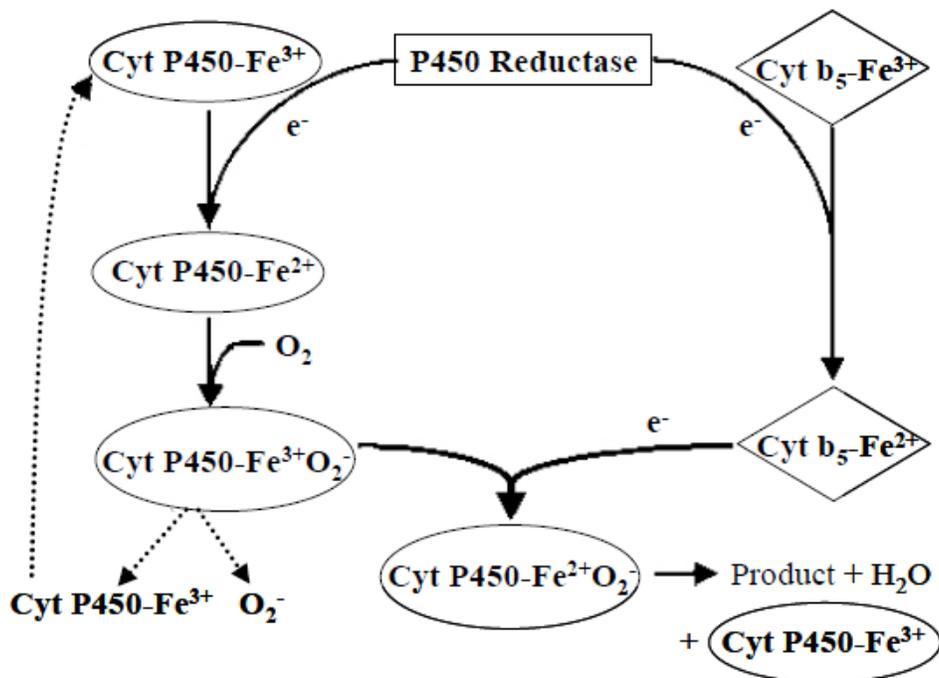
Figure 2.3: Schematic representation of coupling enhancement by cytochrome b5. The solid lines show the path the reaction would follow if cytochrome b5 is present (coupling) and the dashed line depicts the superoxide formation in absence of cytochrome b5 (uncoupling) [1].

2.5.3 Formation of a two-electron acceptor complex with cytochrome P450

As mentioned previously, there are two electrons needed for monooxygenase reactions catalysed by cytochrome P450 and it is accepted that cytochrome b5 provides the second, rate limiting electron. Although cytochrome b5 can accept an electron from NADPH-CPR, this is at a much slower rate than from its natural partner NADH-cytochrome b5 reductase [1]. Furthermore, Lu *et al.* [39] showed that NADH failed to further enhance the metabolism of chlorobenzene by cytochrome P450. This suggested that the supply of the rate limiting electron is not the problem.

Cytochrome b5 is able to form a hetero-complex with both redox partners, CPR and cytochrome b5 reductase, which is made possible through charge pairing [40]. Cytochrome b5 can also form these hetero-dimeric complexes with various cytochrome P450's, and it was shown that, when covalently linked, the complex is still fully functional [1]. In addition, when cytochrome b5 was covalently bound to cytochrome P450 2B4, NADH-cytochrome b5 reductase was unable to reduce cytochrome b5. This suggested that interaction of cytochrome b5 with redox partners, when bound to cytochrome P450's, is blocked [41]. Knowing that

when cytochrome b5 is complexed with cytochrome P450 it still is a functional complex, but also that cytochrome b5 is unable to accept electrons when complexed, it was postulated that the cytochrome b5-cytochrome P450 complex forms a two electron acceptor complex capable of accepting two electrons from a single interaction from CPR. As shown in figure 2.4, cytochrome b5 interacts with cytochrome P450 through charge pairing. The latter enzyme can be readily reduced by NADPH-CPR to its ferrous state. This electron can immediately be passed to cytochrome b5 making cytochrome P450 available to accept a second electron from its redox partner. The complex, together with its two electrons, dissociates from the CPR and reacts with oxygen having the second electron immediately available from cytochrome b5.
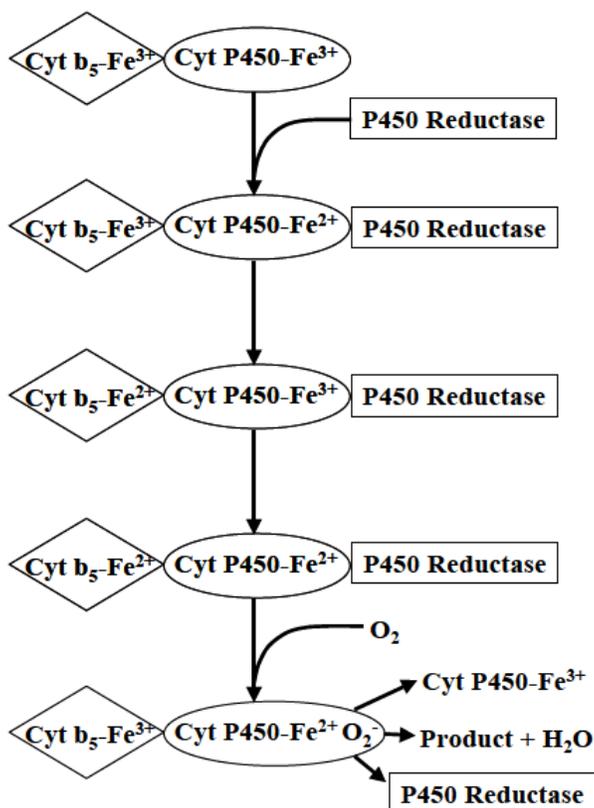
Figure 2.4: Schematic representation of cytochrome b5 and cytochrome P450 forming a two-electron acceptor complex. In a single interaction with CPR two electrons can be passed to the hetero-dimeric complex [1].

2.5.4 Structural influences caused by cytochrome b5 binding

During the metabolism of xenobiotics cytochrome b5 can stimulate these reactions through electron transfer in a number of these oxidative reactions. Moreover, it has been shown that

cytochrome b5 can influence monooxygenase reactions without the transfer of a single electron. For instance, during the metabolism of steroids within the adrenal cortex, cytochrome b5 stimulates the 17,20 lyase activity of P450c17 [42]. Auchus *et al.* [42] showed that the *apo*-cytochrome b5, cytochrome b5 void of heme and incapable of transferring electrons, was as effective as *holo*-chytochrome b5. Similarly, in experiments using cytochrome P450 3A4, Imaoka *et al.* [43] came to the same conclusion which was that the binding of cytochrome b5 to certain cytochrome P450 enzymes causes structural influences which impacts on the ability of the monooxygenase reaction to undergo redox changes. More recently, Goosen *et al.* [44] provided evidence which shows that cytochrome b5 is not only an effector for cytochrome P450's, but also for the enzyme 3βHSD. The investigators showed that *apo*-cytochrome b5 was able to augment the activity of the enzyme. Cytochrome b5 is, however, also known to be a heme scavenger which led the author to produce a mutant variant which was incapable of binding heme. This mutant could still influence the 3βHSD activity and the authors concluded that cytochrome b5 had an allosteric role rather than an electron transfer role in the augmentation of 3βHSD-activity [44]. Figure 2.5 shows a schematic representation of the role cytochrome b5 might play in direct allosteric interactions with cytochrome P450.
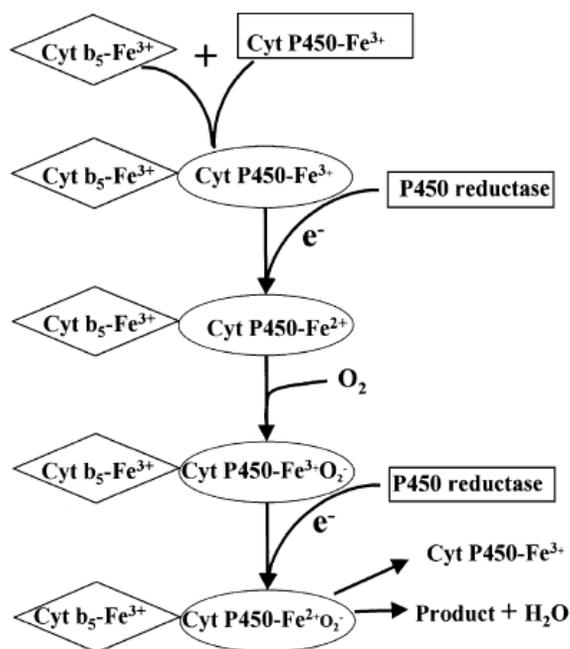
Figure 2.5:   Schematic representation of the role of cytochrome b5 as an allosteric effector. When bound to cytochrome P450 it causes a conformational change which can facilitate in product formation [1].

2.6 Functions of Cytochrome b5

2.6.1 Role in steroidogenesis

2.6.1.1 Cytochrome b5 and 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase

During adrenal steroid metabolism, metabolites of the Δ5-pathway are converted to the products of the Δ4-pathway by the enzyme 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3βHSD). These metabolites pregnenolone and dehydroepiandrosterone are converted to progesterone and androstenedione [45] and 17-hydroxypregnenolone to 17-hydroxyprogesterone [46] indicating that 3βHSD plays a crucial role in adrenal steroid output.

As previously discussed, it has long been known that cytochrome b5 can either enhance reactions through direct electron transfers or as an allosteric effector. Recently, our laboratory showed that cytochrome b5 has an effect on the activity 3βHSD. Goosen *et al.* [44] found that cytochrome b5 augments the activity of 3βHSD. They showed that cytochrome b5 increased the conversion of all metabolites of the Δ5-pathway to their respective products in the Δ4-pathway. It was postulated that the method of augmentation is not through electron transfer but through an allosteric effect as *apo*-cytochrome b5 had a stimulatory effect on the activity of 3βHSD. In addition, this stimulatory effect was not due to a random, non-specific membrane effect. Stimulation of 3βHSD-activity was also achieved using the truncated version of the protein, a mutation which contained a shortened C-terminus and therefore lacking the membrane anchoring domain. This finding together with the fact that 3βHSD does not need any electrons from an external electron donor, led the authors to suggest that the augmentation was due to an allosteric effect of cytochrome b5 on 3βHSD [44][46], like in the case with P450c17 [42].

Recently, additional evidence was reported for the augmentation of 3βHSD activity by cytochrome b5. Fluorescence resonance energy transfer (FRET) studies revealed that the co-expression of 3βHSD and cytochrome b5 fluorescent constructs had a significant increase in the FRET signal. This finding suggests that the these two enzymes indeed formed hetero-dimeric complexes further confirming that the augmentation was due to an allosteric interaction [46].

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Goosen *et al.* [46] went on to demonstrate that, using purified enzymes, the presence of cytochrome b5 increased 3βHSD's affinity for NAD^+, the cofactor needed for the activity for the dehydrogenase reaction of the enzyme [47]. During the metabolism of Δ5-metabolites, the substrate together with NAD^+ binds to 3βHSD resulting in the formation of a $\Delta^{5\text{-}3}$ ketosteroid intermediate and NADH; both of which remain bound to the enzyme. NADH causes a conformational change in the enzyme which results in the isomerisation of the intermediate [48]. When cytochrome b5 was added the K_m decreased more than threefold and the presence of the hemoprotein also increases the $V_{max \; apparent}$ by 17%. These data suggested that cytochrome b5 increased the 3βHSD affinity for its cofactor NAD^+ [46].

2.6.1.2 Cytochrome b5 and Cytochrome P450c17

During adrenal steroidogenesis cholesterol is converted pregnenolone by the cytochrome P450 11A1, commonly known as P450 side chain cleavage. As discussed earlier, pregnenolone serves as a substrate for two enzymes: 3βHSD and cytochrome P450c17 (P450c17). The former is responsible for the conversion of pregnenolone to progesterone and the latter is a multifunctional enzyme having both a hydroxylase and a lyase activity. During its hydroxylase activity, P450c17 introduces a hydroxyl group at the 17α-position of 21-carbon steroids such as pregnenolone and progesterone resulting in the production of 17-hydroxy-pregnenolone (17α-OH-PREG) and 17-hydroxy-progesterone (17α-OH-PROG), respectively. Moreover, both the products of the hydroxylase reaction are substrates for the 17,20-lyase reaction catalysed by the same enzyme, P450c17. During this reaction, P450c17 catalyses the 17,20-lyase reaction by cleaving the $C_{17} – C_{20}$ bond of 17α-OH-PREG and 17α-OH-PROG forming C_{19} sex steroid precursors, dehydroepiandrosterone (DHEA) and androstenedione (A4). This 17,20-lyase activity takes place at a much slower rate than its hydroxylase activity [49].

Although this reaction is slow compared to the hydroxylase activity, it has been shown that cytochrome b5 augments the 17,20-lyase reaction [50] without affecting the hydroxylase activity of P450c17 [10]. Furthermore, the 17,20-lyase activity is known to be relevant to the androgen production. Auchus *et al.* [10] showed that by expressing P450c17, in *Saccaromysces cerevisiae*, without its redox partner, both the hydroxylase and 17,20-lyase activities could be observed with the latter to a lesser extent. Upon the addition of its redox partner they found that the V_{max} of both reactions increased more than fivefold. On co-expression of human cytochrome b5 the authors found that cytochrome b5 only increases the

V_{max} of the 17,20-lyase activity of P450c17 without having any effect on its hydroxylase activity. The same effect was observed when adding purified cytochrome b5 to a microsomal preparation. When adding *apo*-cytochrome b5, a form of the protein void of heme and incapable of participating in electron transfer reactions, a similar effect could be observed. Furthermore, Lee-Robichaud *et al.* [51] found that using Mn^{2+}-substituted cytochrome b5 the 17,20-lyase activity could be stimulated two to fourfold; even though this form of the protein stays almost completely in the oxidised form in the presence of both NADPH:CPR and NADH:cytochrome b5 reductase. Omitting the option that the stimulation of cytochrome b5 is through electron transfer, it is to date generally accepted that cytochrome b5 enhances the 17,20-lyase activity of P450c17 allosterically [42]. Similar allosteric roles of this protein have been reported, examples include its affect on cytochrome P450's 2A6, 2B6, 3A4 [52] and more recently 3βHSD [44].

A second form of cytochrome b5 has been characterised [53]. Cytochrome b5 *type 2* shared about 45% sequence identity and was an additional 15 amino acids longer than the full-length cytochrome b5. This addition was noted at the amino terminus. Soucy [53] reported that, in a reconstituted system, the 17,20- lyase activity of P450c17 could also be stimulated by the addition of this *type 2* cytochrome b5. It was also reported that this type of cytochrome b5 is not only expressed in the liver, but in both the adrenal glands and the testis.

2.6.2 Ellipticine metabolism is altered by cytochrome b5

Ellipticine is a drug which is taken orally. Once ingested, the alkaloid is taken up in the intestine and delivered to the liver; the organ responsible for metabolism of foreign molecules, where after it is metabolised by cytochrome P450 enzymes to a number of derivatives. Some of the resulting metabolites are efficient anticancer compounds capable of forming highly reactive carbonium ions which can further react resulting in the formation of DNA adducts [54]. These adducts are predominantly responsible for targeting and killing cancer cells through apoptosis and cell cycle arrest. Cell cancers include: leukemias, lymphosarcomas, lung carcinomas and breast adenocarcinomas [55] [54].

Metabolised by cytochrome P450 3A4, the metabolites formed are 13-hydroxyellipticine, 12-hydroxyellipticine and ellipticine-N^2-oxide [54]. The latter metabolite can form 12-hydroxyellipticine through Polonovski rearrangement. The 12 –and 13-hydroxyellipticine can readily dissociate to ellipticine-12-ylium and ellipticine-13-ylium respectively, which in turn are able to bind to guanine of DNA forming deoxyguanosine adducts [54]. The reaction for

the production of these two major products can also be catalysed by enzymes cytochrome P450 1A1 and/or 2 [54].

Recently it has been reported that cytochrome b5 not only alters the amounts of metabolites produced but also the type of metabolites formed [56]. Cytochrome P450 1A1/2 are capable of either detoxifying ellipticine forming 7– and 9-hydroxyellipticine. These metabolites are incapable of forming DNA adducts leading to a lower efficacy of the drug. The authors showed that, in the presence of cytochrome b5, the major metabolite observed was 13-hydroxyellipticine. As mentioned previously, this metabolite can further react and form DNA adducts [56]. The same observation was made by Frei *et al.* [55] who reported that DNA adduct formation was enhanced by cytochrome b5. Cytochrome b5 was then suggested of being capable of switching certain cytochrome P450 enzymes from detoxification of ellipticine to formation of more active metabolites, in turn causing apoptosis, therefore making cytochrome b5 a modulator of the efficiency of the anticancer drug [56].

 The mechanism by which cytochrome b5 modulates the activity of these cytochrome P450 enzymes is not clearly understood. Studies by Kotrbová *et al.* [56] found that *holo*-cytochrome b5 had a more significant effect than *apo*-cytochrome b5. Furthermore, using manganese substituted cytochrome b5 yielded similar results as that of the *apo*-form, suggesting that enhancement could be due to the input of the rate-limiting electron. What is important to note, is that it has been reported that *apo*-cytochrome b5 and Mn-protoporphyrin IX co-exists in solution [57]. The investigators did not dismiss a structural role of cytochrome b5, the reason being that *holo* –and *apo*-cytochrome b5 differ in three dimensional structure. The former consisting of four helices and three loops while the latter only contains a single helix. This causes significant structural changes in the surface geometry of cytochrome b5 preventing adequate protein-protein interaction. It was suggested that more studies regarding this matter should be completed [56].

2.6.3 Reduction of methaemoglobin by cytochrome b5

Haemoglobin is a hemoprotein responsible for carrying molecular oxygen from respiratory organs to the rest of the body. During the day a small percentage of oxygen-binding haemoglobin is oxidised to non-functional methaemoglobin [58]. Soluble cytochrome b5 is a smaller form of cytochrome b5 lacking the membrane binding domain. Although it is structurally different from the full-length form, the two proteins are functionally similar; exhibiting the same absorption spectra [59].

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Abe and Sugita [60] reported the midpoint potential of the soluble form of cytochrome b5 to be -2 mV. Despite this relatively low redox potential and low abundance cytochrome b5 could sufficiently reduce methaemoglobin. It was reported that the soluble form of the protein had a similar affinity for its redox partner than that of full-length cytochrome b5 solubilised using trypsin. The optimum pH for this reduction was found to be around 5.5 under aerobic conditions. The presence of cytochrome b5 increased the rate of reduction of methaemoglobin considerably. However, what was observed was that at a neutral pH this reduction potential declined significantly. Furthermore, it was reported that this reduction was dependent on the presence of cytochrome b5 reductase. In the absence of this redox partner no reduction of methaemoglobin was observed [60].

2.6.4 Lipid Biosynthesis

Cytochrome b5 is involved in lipid biosynthesis through its interaction with desaturases; the enzymes responsible for synthesizing unsaturated fatty acids [1]. These fatty acids are found not only in animals, but also in plants and bacteria. Animals use the aerobic pathway for the synthesis of these fatty acids and cytochrome b5 donates a single electron to these desaturases. These enzymes utilise this electron to generate an electron-deficient, activated oxygen species which in turn reacts with saturated hydrocarbons, removing electrons from them. There are a number of desaturases which are involved in synthesising different unsaturated fatty acids which serve a structural role in membrane fluidity. A few examples of these desaturases are Δ^9-, Δ^6-, Δ^{12}- and $\Delta 5$ desaturases. The Δ^9 desaturases are the most completely characterised while the $\Delta 5$ desaturases are the least characterised and each functioning through the insertion of a double bond into their respective positions, given in their names [1].

Phosphoglycerides, better known as plasmalogens, are vinyl-ether linked fatty alcohols which make up 20% of the phospholipid mass in humans alone. The final step in the biosynthesis of plasmalogens involves the introduction of a *cis*-double bond forming a vinyl-ether functionality. The enzyme, plasmanylethanol-amine Δ-desaturase, is responsible for this catalysis and is totally dependent on cytochrome b5 [1].

2.6.5 Cholesterol Biosynthesis

During the biosynthesis of cholesterol, lanosterol is converted to steroid-4α-oic acid through the oxidation of the C-30 methyl group. The enzyme responsible for the oxidation reaction is

4-methyl sterol oxidase. This enzyme functions through the oxidation of the C-30 group of a cholesterol precursor. Once oxidised to a steroid-4α-oic acid, it can subsequently be decarboxylated [9]. Cytochrome b5 provides reducing equivalents to the sterol oxidase for functionality. Upon the addition of anti-cytochrome b5 immunoglobulins it was observed that 4-methyl sterol oxidase was inhibited by up to 50% [1]. It was demonstrated, in a reconstituted microsomal system, that when cytochrome b5 is treated with trypsin the enzyme's oxidase activity is abolished. The oxidase activity, however, was completely restored after the same trypsin treated microsomes were washed and purified cytochrome b5 was added to the same reconstituted system. This evidence suggested that the membrane bound form of cytochrome b5 is an obligatory electron carrier to microsomal 4-methylsterol oxidase [61].

Another precursor in cholesterol biosynthesis is Δ7-cholesterol (lathosterol). The enzyme lathosterol Δ5-desaturase catalyses the formation of a double bond between carbons positioned at numbers 5 and 6, producing Δ^7-dehydrocholesterol. Grinstead and Gaylor [62] isolated this enzyme chromatographically using different stationary phases. Using a DEAE-cellulose resin for purification of detergent solubilised microsomal suspension yielded a fraction which contained the desaturase, phospholipid and cytochrome b5 reductase. Activity of this enzyme was only noticed after introduction of cytochrome b5 to this fraction. An additional chromatographic purification step of this fraction, using Sepharose CL-4B resin, resulted in a fraction containing only the desaturase and cytochrome b5 reductase. This fraction showed activity when both cytochrome b5 and the phospholipid were added. The authors concluded that for optimal activity of lathosterol Δ5-desaturase both cytochrome b5 and its redox partner were necessary [62].

2.6.6 The effect of Cytochrome b5 on other Cytochromes P450

In literature it is known that cytochrome b5 can stimulate, inhibit or have no effect on P450 enzymes. It does this either through delivering the second, rate limiting electron during monooxygenase reactions or via an allosteric interaction [1]. Cytochrome b5 is known to effect up to 20 different P450 enzymes and a few examples will be discussed.

2.6.6.1 Cytochrome P450 2B4

Xenobiotics such as cyclohexane and benzphetamine are metabolised by the P450 enzyme, cytochrome P450 2B4, to cyclohexanol and norbenzphetamine respectively. It was initially

believed that cytochrome b5 stimulates the activity of this enzyme through donation of the second electron [63], but further studies have suggested that both the electron donating and effector function of cytochrome b5 enhances the activity of cytochrome P450 2B4 [64]. Zhang *et al.* [65] reported that, under steady-state conditions, cytochrome b5 not only increases the rate of product formation, but also competes with cytochrome P450 reductase for binding on the P450 enzyme. It is hypothesised that if cytochrome b5 competes with CPR for binding, it would be observed in the decrease in NADPH consumption resulting in a decrease in product formation. This was indeed the case. By having fivefold more cytochrome b5 than CPR, NADPH consumption and product formation was depleted more than 90%. Furthermore, the rate of oxidation of benzphetamine to norbenzphetamine, in the presence of cytochrome b5 alone, was found to be 18 s^{-1}, whilst in the presence of CPR it was about 100-fold slower; 0.15 s^{-1} [65].

2.6.6.2 Cytochrome P450 2C9

Another member of the P450 enzyme family which will be discussed is cytochrome P450 2C9. In a reconstituted system containing cytochrome P450 2C9 and CPR the oxidation of substrates was compared with an increase in the amount of cytochrome b5. Using three different substrates, more than a 100% increase in product formation could be observed with a molar ratio of cytochrome b5 to cytochrome P450 2C9 of about 4. An increase in this ratio, to more than 4, resulted in a rapid decline in product formation. At ratios above 16, levels of product formation were almost completely depleted and the same as when no cytochrome b5 was added [66]. During monooxygenase reactions, if a second electron is not supplied rapidly the substrate could dissociate from the P450 enzyme resulting in the release of superoxides, which can further dismutate to hydrogen peroxide [1]. During catalyses by cytochrome P450 2C9, hydrogen peroxide formation was also significantly decreased when cytochrome b5 was present. A maximum decrease was observed at ratios where maximum product formation was observed. These findings suggested that not only did cytochrome b5 affect the catalyses of the P450 enzyme but that the amount of cytochrome b5 present also plays a vital role [66].

2.6.6.3 Cytochromes P450 3A4 and 3A5

These two P450 enzymes comprise around 50% of all cytochrome P450 enzymes within the liver [67]. Both of the enzymes are capable of metabolising testosterone and are highly depended on both CPR and cytochrome b5 for optimum activity [52]. Recently, Lee and Goldstein [68] did a comparative study between these two enzymes in the metabolism of

testosterone. They found that for the activity of the P450 enzymes was highly dependent on the amount of CPR present. For both enzymes, the metabolism of testosterone increased as the ratio P450 enzyme to CPR increased. This observation had a similar pattern for cytochrome P450 3A4/5. The activity increased to a ratio of about four to eight, while at higher ratios the activity gradually decreased. For all these experiments, cytochrome b5 was kept constant. To determine whether the amount of cytochrome b5 had an effect on the activity, the concentration was increased at the calculated optimum ratio of P450 to CPR. The authors reported that as the amount of cytochrome b5 was increased, so did the activity. This increase was up to a certain amount, where after the activity gradually declined. For both enzymes the highest activity was observed for a cytochrome b5 at the same molar amount (P450:b5:CPR 1:16:8 and 1:16:4 for 3A4 and 3A5 respectively). Their study showed that the activity of these enzymes are both influenced by cytochrome b5 [68].

2.7 Summary

Cytochrome b5 is a protein that has been discovered a number of decades ago and has been shown to be involved in a number of reactions. It has also been shown that it plays a pivotal role during steroidogenesis by influencing the activity of at least two enzymes during steroid production. The postulated mechanisms of action for cytochrome b5 show the complexity of interactions between this protein and cytochromes P450 and more studies are needed to understand these hypotheses. In order to complete this study, cytochrome b5 was isolated from ovine liver microsomes. The reason for this was that the commercially available form of the protein is relatively expensive and large quantities were needed. The subsequent chapter discusses how the membrane bound protein was purified.

Chapter 3

3. Isolation of Cytochrome b5 from ovine liver microsomes

3.1 Introduction

Cytochrome b5 is commercially available at certain chemical reagent suppliers. The recombinant form of the protein is, however, relatively expensive and supplied in µg amounts only. For this study, considerable amounts of cytochrome b5 were needed and due to the cost of commercial cytochrome b5 the protein had to be isolated from animal tissue.

Cytochrome b5 was previously isolated from animal tissue [4] including ovine liver microsomes [3]. The hemoprotein exists as two forms, namely a soluble form, found primarily in the blood, and as a membrane bound form found in organs such as the liver and testes [9]. These two forms differ from one another in structure. The soluble form of the protein lacks the membrane anchoring region and has a reported molecular weight of *ca.* 11 kDa [3], while the membrane bound form contains the membrane binding region and has a reported molecular weight of 15.2 kDa, excluding the heme group [6]. Storbeck *et al.* [6] reported that cytochrome b5, isolated from animal tissue, was able to form multimeric complexes and that the intact cytochrome b5 not only exists *in vivo* as a 16 kDa monomer, but also as homomeric complexes.

In order to further investigate these findings, cytochrome b5 had to be isolated from ovine liver microsomes. In this chapter the purification of cytochrome b5 from ovine liver is described. This includes the preparation of liver microsomes and subsequent solubilisation of membranes. Cytochrome b5 was purified using three DEAE chromatography steps which were monitored by spectral analyses for the presence of cytochrome b5. The purified protein was subsequently subjected to SDS-PAGE and Western blot analyses, using antibodies previously raised in rabbits against cytochrome b5, to assess purity. Finally, cytochrome b5 was subjected to ESI-MS for accurate weight estimation of the protein.

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3.2 Experimental Procedures

3.2.1 Preparation of ovine liver microsomes

Ovine liver was collected from freshly slaughtered sheep from a local abattoir and liver microsomes were prepared as described by Yang and Cerderbaum. [4]. All experiments were performed at 4°C. The liver was washed with a 1.15% (*m/v*) KCl solution where after all connective tissue and excess fat was removed. The organ was cut into small pieces and placed in cold 10 mM TRIS-HCl buffer, pH 7.4, containing 1.0 mM EDTA and 0.25 M sucrose. The preparation was subsequently homogenised in a Waring blender at low speed, followed by homogenisation with a dounce homogeniser. This homogenate was centrifuged for 10 minutes at $500 \times g$ where after the resultant supernatant was centrifuged again for 16 minutes at $11\ 000 \times g$ and the post mitochondrial fraction was collected. To prepare the microsomes, a 50% (*m/v*) polyethylene glycol 8000 (PEG) solution was added to the post mitochondrial fraction to a final PEG concentration of 8.5% (*m/v*) while stirring slowly. This mixture was stirred for an additional 10 minutes and centrifuged for 20 minutes at $13\ 000 \times g$ to pellet the microsomes. The pellet was resuspended in 10 mM TRIS-HCl buffer, pH 7.4, containing 1.0 mM EDTA and 150 mM KCl using a dounce homogeniser. Again, PEG was added to this suspension to a final concentration of 8.5% (*m/v*) and stirred for 10 minutes. The suspension was centrifuged for 20 minutes at 13 000 x g. This procedure was repeated 4 times and the final pellet was suspended in 10 mM TRIS-HCl buffer, pH 7.4, containing 1.0 mM EDTA and 0.25 M sucrose. An aliquot was removed and the rest of the microsomal preparation was stored at −80°C until further use.

3.2.2 Determination of protein content

The aliquot of prepared microsomes were thawed on ice and the total protein content was determined using a bicinchoninic acid (BCA) method. The method was followed as described according to the manufacturer's instructions, using a dilution series of bovine serum albumin (BSA) as protein standard with concentrations ranging from 0.4 mg.mL^{-1} to 2.0 mg.mL^{-1} [69].

3.2.3 Solubilisation of microsomal membranes

Prior to chromatographic separation, the microsomes were solubilised using Triton X-100 as described by Yang and Cederbaum [4]. In brief, the protein concentration of the microsomes

was determined using a BCA protein determination kit as described in section 3.2.2. The microsomes were then diluted to a concentration of ~8 mg protein/mL with 0.1 M TRIS-acetate buffer, pH 8.1, containing 1 mM EDTA. A 10% (*v/v*) stock solution of Triton X-100 was added to a final Triton X-100 concentration of 2% (*v/v*) and the resulting solution was allowed to stir at 4°C. After 60 minutes, PEG was added to the suspension to a final concentration of 8.5% (*m/v*) and stirred for an additional 30 minutes and subsequently centrifuged for 25 minutes at 13 000 × *g*. The resulting yellowish supernatant contained all previously membrane bound proteins, including cytochrome b5.

3.2.4 Determination of cytochrome b5 content in ovine liver microsomes

The concentration of cytochrome b5 within the prepared microsomes was determined spectrophotometrically using a Beckman DU 650 spectrophotometer [27]. The solubilised microsomes were diluted 10-fold using phosphate buffered saline (PBS) and 1 mL of the diluted sample was placed in each cuvette inside the cell compartment. A baseline is recorded between 600 and 350 nm, to compensate for the difference in light absorbance between the sample cuvette and the reference cuvette. Once this baseline was established, cytochrome b5 in the sample cuvette was reduced by the addition of NADH and a cytochrome b5 reduced difference spectrum recorded between 600 and 350 nm. The concentration of cytochrome b5 was calculated from the reduced difference spectra using a molar extinction coefficient (ε) of 185 $mM^{-1}.cm^{-1}$ and the absorbance change at 426 nm minus the absorbance at 409 nm.

3.2.5 Ion exchange column chromatography of solubilised microsomes

Cytochrome b5 has a reported iso-electric point of ~4.4 [70]. Working at a physiological pH would thus mean the protein would carry a net negative charge. For purification of cytochrome b5, a suitable matrix bearing a positive charge was therefore used for the isolation of the protein of interest.

Cytochrome b5 was isolated using a three step chromatographic procedure [71]. All chromatographic procedures were carried out at 4°C. The solubilised microsomes were thawed overnight at 4°C and applied to a DEAE-cellulose column (3.2 × 20 cm) equilibrated with 80 mM TRIS-acetate, pH 8.1, containing 1 mM EDTA and 2% Triton X-100 (equilibration buffer A). After sample application, all unbound proteins were washed from the column with at least three bed volumes of equilibration buffer A. Cytochrome b5-reductase was subsequently eluted with equilibration buffer A. The column was conditioned

with a linear gradient of NaSCN (0 – 50 mM), in elution buffer A, over three column volumes followed by the elution of cytochrome b5 with 90 mM NaSCN in elution buffer A. From the collected fraction, a 1 mL aliquot was stored at −20°C and the remainder subsequently dialysed overnight against 10 sample volumes of 10 mM TRIS-acetate buffer, pH 8.1 using a Spectra/Por® Dialysis Membrane.

After dialyses, the dialysate was applied to a second DEAE column (2 × 20 cm) previously equilibrated with elution buffer A, containing 0.05% deoxycholic acid (equilibration buffer B). After sample application, the column was washed with 3 bed volumes of equilibration buffer B followed by a linear gradient from 0 – 50 mM NaSCN in the same buffer over three column volumes. The cytochrome b5 was eluted with a linear gradient from 50 – 90 mM NaSCN over five column volumes. Again an aliquot was taken, stored at −20°C and the eluted protein was dialysed in the same manner as mention before against 10 mM TRIS-acetate, 0.1 mM EDTA, pH 8.1 (equilibration buffer C).

To concentrate and remove the detergent from the dialysate, the cytochrome b5 preparation was loaded on a third, smaller, DEAE column (1 × 5 cm) equilibrated with the equilibration buffer C. The bound protein was then washed with at least 2.5 L of equilibration buffer C, whereafter the cytochrome was eluted with equilibration buffer C, containing 0.25 M NaSCN and 0.25% deoxycholic acid. Another aliquot was taken from the eluate before it was subjected to extensive dialyses against equilibration buffer C.

3.2.6 Determination of total protein content in purified fractions

Total protein content of all collected fractions was determined using the BCA method previously described in *section 3.2.2*.

3.2.7 Determination of cytochrome b5 content

Cytochrome b5 content within each of the three eluates was determined spectrophotometrically by the method described by Estabrook and Werringloer [27]. An appropriate dilution of the cytochrome b5 fractions was made using PBS buffer and an absolute spectrum of cytochrome b5 was recorded between 600 and 380 nm, using PBS buffer as a reference. The cytochrome b5 was subsequently reduced in the sample cuvette by the addition of sodium-dithionite. The reduced cytochrome b5 spectrum was recorded between 600 and 380 nm and the concentration of cytochrome b5 was determined by

applying a molar coefficient of 100 000 M^{-1}cm^{-1} using the difference in absorbance at 424 nm between the reduced and the oxidised spectra.

3.2.8 SDS-PAGE analyses of cytochrome b5

All purified fractions and solubilised microsomes were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses according to the method of Laemmli [72].

3.2.8.1 Preparation of separating gel and stacking gel

To prepare the separating gel, 6.71 mL TRIS-HC*l* buffer, (0.56 M) pH 8.8, was mixed with 100 μL 10% SDS, 5 μL *N,N,N,N*-tetramethylenediamine (TEMED) and 3.14 mL acrylamide/bisacrylamide (40% stock) solution. The polymerisation reaction was initiated by the addition of a freshly prepared 10% (*m/v*) ammonium persulfate, 50 μL. The mixture was poured, within a minute of initiation, between two glass plates spaced 1.5 mm apart. Time allowed for polymerisation was 45 minutes. For the stacking gel, 4.47 mL TRIS-HCl buffer, (0.14 M) pH 6.8, was mixed with 50 μL 10% SDS, 5 μL TEMED and 0.5 mL acrylamide/bisacrylamide (40% stock) solution. The polymerisation reaction was initiated by the addition of 25 μL 10% ammonium persulfate. This solution was immediately poured on top of the separating gel and a 10-well comb was carefully inserted. After 30 minutes the glass plates were wrapped in moist tissue paper and the gel was stored overnight at 4°C.

3.2.8.2 Sample preparation for SDS-PAGE analyses

In order for the separation of the proteins according to their molecular mass it needs to be treated with SDS, to not only denature proteins but also yield a same charge-to-mass ratio for all proteins present, and β-mercaptoethanol to break all disulfide bonds. Samples were denatured by incubating with equal volume Laemmli sample buffer$^{®}$ (67.5 mM TRIS-HCl buffer, pH 6.8, 2% SDS, 10% glycerine and 5% β-mercaptoethanol) at 90°C for 10 minutes, whereafter it was kept at 4°C until further use.

3.2.8.3 Gel electrophoresis of cytochrome b5

 The proteins (2 – 10 μg) were loaded and separated using a BioRad MINI Protean system. The tank and inner chamber were filled with 25 mM TRIS-HCl buffer (pH 8.3), containing 0.25 M glycine and 0.1% SDS. Separating conditions were 200 V for 60 minutes at 4°C. After protein separation, the gel was stained using a Coomassie Brilliant Blue R-250 staining

solution (3 mM Coomassie R-250, in 10% acetic acid and 45% methanol) for 60 minutes where after it was placed in destain solution 1 (50% (*v/v*) methanol, 10% (*v/v*) acetic acid) overnight. Rehydration of the gel was accomplished by placing it in destain solution 2 (20% (*v/v*) methanol, 7% (*v/v*) acetic acid).

3.2.9 Immunoblotting of cytochrome b5

3.2.9.1 *Western blot analyses of cytochrome b5 using anti-cytochrome b5 immunoglobulins*

A Western blot analysis was also performed on separated proteins, after SDS-PAGE. The proteins were transferred to nitrocellulose membrane (BioTrace^{TM} NT – Life Sciences) using a wet transfer system (transfer buffer: TRIS, (0.05 M), pH 8.3, 0.2 M Glycine and 20% (v/v) MeOH). A BioRad transfer system was used and the proteins were transferred at 110 volt for 90 minutes at 4°C. The efficiency of the transfer was confirmed by the presence of the KaleidoScope rainbow marker used, as it could be visualised on the membrane without any additives. The resulting membrane was blocked by allowing the membrane to soak in casein buffer (10 mM TRIS, pH 7.6, 0.15 M NaCl, 0.5% (*m/v*) casein) for 30 minutes at 37°C with gentle agitation. This was followed by incubating the blocked membrane in a rabbit-anti-cytochrome b5 antibody solution (1: 1 000 in blocking buffer) at 37°C. After 60 minutes the antibody solution was decanted and the membrane was washed 3×5 minutes with PBS – Tween-20 (PBS containing 0.1% Tween-20). After the wash step, the membrane was incubated with a previously diluted solution of goat-anti-rabbit secondary antibody (1: 20 000 in blocking buffer), conjugated to horseradish peroxidase (HRP) (Sigma-Aldrich, St. Louis, USA). After 60 minutes at 37°C the membrane was once again washed as described earlier.

3.2.9.2 *Visualisation of immobilised protein*

The immobilised protein was subsequently visualised by chemiluminesence. This was accomplished by incubating the membrane in a substrate solution (Pierce ECL Western Blotting substrate), prepared as described by the manufacturer's instructions, for 1 minute and images were developed on X-ray film (Super RX – Fuji Medical X-Ray Film) with a minimum of 30 seconds exposure time.

3.2.10 ESI-MS analyses of cytochrome b5

A technique commonly used for the accurate mass determination of biomolecules such as proteins is electrospray ionisation mass spectrometry (ESI-MS). During this process, particles, after a separation step, are ionised by using one of a number of known ionisation steps known. After ionisation, the charged particles move through a detector which detects the mass-to-charge ratio, which in turn can be used to determine the mass of the particle [73]. Prior to ESI-MS analysis, the sample should be free of high ionic strength buffers, detergents, etc, as these substances could interfere with the ionisation process which could lead to false results [74].

As we have isolated the membrane bound cytochrome b5 using Triton X-100 it was necessary to determine the quantity within the final solution.   The remainder of the detergent was removed using a Pierce® Detergent Removal Spin Column according to the procedure described by the manufacturer. In brief: after centrifugal removal of the storage solution, the column was washed/equilibrated using 400 µL PBS. The PBS was added on top of the column and the column was centrifuged $1500 \times g$ for 1 minute. This step was repeated 3 times before 100 µL of protein solution was applied slowly to the column, incubated for 2 minutes at ambient temperature and the protein solution centrifuged for 2 minutes at $1500 \times g$. The protein solution obtained was immediately collected by placing in a 200 µL glass vial insert and sealed for ESI-MS analyses. The amount of detergent within the final dialysed fraction was determined spectrophotometrically. This was accomplished by using a standard curve created from a dilution series of detergent and the absorbance spectra from 260 to 300 nm was measured (Cary Beckman DU 650). The fourth derivative spectra were calculated and plotted using the accompanied software (Cary Beckman UV-100 software). A standard curve was set up using a dilution of Triton X-100 (0.05% − 0.01%) in 1 M KOH. To determine the amount of Triton X-100 left in the dialysate, the cytochrome b5 sample was diluted 10-fold in 1 M KOH as the pH of this preparation is above 12 and all tyrosine residues are fully dissociated and would have an insignificant influence on the absorbance [75].

The accurate mass of desalted detergent free cytochrome b5 was determined with the use of ESI-MS. The protein solution was delivered to a Waters Synapt G2 instrument using a Waters Acquity UPLC® delivering a solvent gradient from 0.1% formic acid to 100% acetonitrile, at 200 µL/min, without the use of a column. The sample was introduced through

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a conventional electrospray probe with a capillary voltage of 3.0 kV and a cone voltage of 20 V. Data were collected in the positive mode by scanning over an *m/z* range of 200-3000.

3.3 Results

3.3.1 Total protein content in prepared microsomes

Cytochrome b5 was prepared from fresh ovine liver microsomes using the PEG fractionation method. A summary of the purification results is given in Table 3.1.

Table 3.1: Total protein content within the solubilised microsomal fraction.

Fraction	Mass of ovine liver (g)	Total Volume (mL)	[Protein] (mg/ml)	Total Protein (g)	Cytochrome b_5 content in microsomes (mM)	Total Cytochrome b_5 (µmol)
Triton X-100 filtrate	800	550	5.91	3.25	1.77	0.97

3.3.2 Determination of cytochrome b5 content in ovine liver microsomes

Cytochrome b5 can be readily reduced using sodium dithionite in the absence of its redox partner cytochrome b5 reductase. In microsomes there are, however, other cytochrome P450 enzymes present which can also be reduced and absorb in the Soret region. The appropriate reducing agent for cytochrome b5 would therefore be NADH. NADH reduces cytochrome b5 reductase, still present in microsomes, which in turn specifically reduces cytochrome b5. A difference spectrum of cytochrome b5 (figure 3.1), with an absorption maximum at 426 nm, was used to determine the molar concentration of cytochrome b5 in the microsomal preparation. Using a molar extinction coefficient of 185 mM^{-1}.cm^{-1} the cytochrome b5 was calculated to be 1.768 mM in the liver microsomal preparation (Tables 3.1 and 3.2).
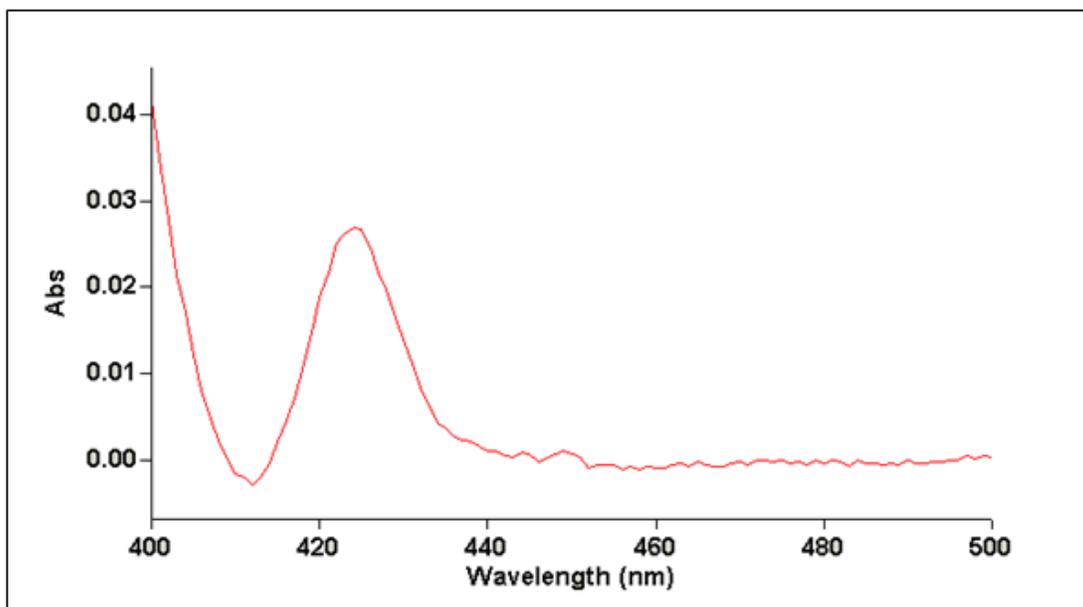
Figure 3.1: Difference spectrum between oxidised and reduced cytochrome b5. The difference in absorbance at 426 nm and 409 nm was used for determining the cytochrome b5 concentration. NADH (2 mM) was used as reducing agent.

3.3.3 Purification of cytochrome b5 using ion exchange chromatography

Cytochrome b5 was purified chromatographically at 4°C. After applying the microsomes to the DEAE cellulose resin cytochrome b5 was separated from its redox partner, cytochrome b5 reductase, which eluted as a yellow band using a high concentration of TRIS-HCl buffer. A linear gradient of sodium thiocyanide was used to elute all impurities before cytochrome b5 was eluted as a red band from the column. The eluate was assayed spectrophotometrically for cytochrome b5 activity (figure 3.2 A). For this assay procedure, cytochrome b5 was reduced with sodium dithionite and cytochrome b5 was identified by the shift in absorbance maxima from 412 nm to 424 nm in the absolute absorption spectra shown in figure 3.2. Furthermore, the reduced spectra also contained additional identification peaks between 550 and 570 nm. Once the presence of cytochrome b5 was established the eluate was dialysed and the procedure was repeated using a smaller column. This additional chromatography steps was not only used for purifying cytochrome b5 from contaminating proteins but also used for concentrating the protein. Figure 3.2 (B and C) represents the aliquots taken from the second and final chromatographic steps.
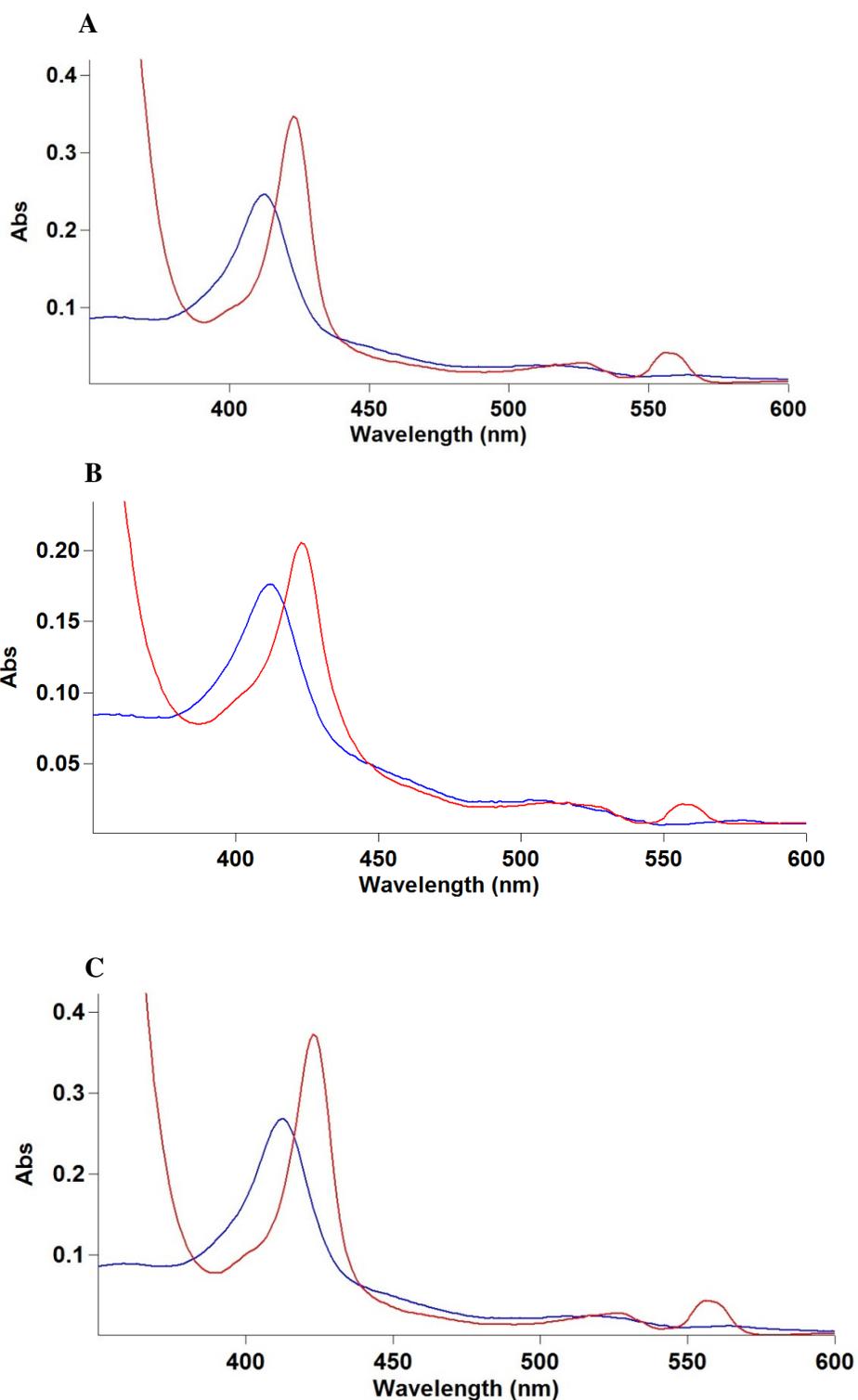
Figure 3.2: Oxidised (blue line) and reduced (red line) spectra of different fractions during cytochrome b5 purification. Fraction from each column; 3.2 × 20 cm (A), 2.0 × 20 cm (B) and 1.0 × 50 (C) cm, was reduced using a few grains of granular sodium dithionite. The shift in maximum absorbance from 412 to 424 nm confirmed the presence of cytochrome b5.

3.3.4 Determining total protein and cytochrome b5 content of purified fractions

The total protein content was determined in each of the fractions using the BCA method with lysozyme as a standard. The cytochrome b5 content in each fraction was determined spectrophotometrically using the difference in absorbance of the reduced and the oxidised spectra at 424 nm and a molar coefficient of 100 000 $M^{-1}cm^{-1}$ to calculate the cytochrome b5 concentration (figure 3.2). The results for total protein and cytochrome b5 concentrations are shown in Table 3.2. This table also contains a detailed set of purification data which were collected throughout the cytochrome b5 isolation procedure.

Table 3.2: Purification table for cytochrome b5 isolated from ovine liver microsomes.

Fraction	Total Volume (mL)	[Protein] (mg/ml)	Cytochrome b_5 (µM)	Total Cytochrome b_5 (µmol)	Specific Activity (µmol/mg)	Fold Purification	Yield (%)
Triton X-100 filtrate	550	5.91	1.77	0.972	0.0003	1.0	100
1st DEAE Eluate	150	0.96	5.92	0.888	0.006	20.7	91.4
2nd DEAE Eluate	87	0.33	6.96	0.606	0.021	69.7	62.3
3rd DEAE Eluate	13	1.16	41.2	0.535	0.035	118	55.1

During this study cytochrome b5 was isolated from ovine liver more than once, using the same procedure as discussed previously. We found, through up-scaling about three times, that the fold purification (for the last step) remained the same, but a similar final yield with a marginally lower specific activity was achieved than the smaller scale purification. Thus it can be said, although the yield is the same the purification is at least 50% lower in the scaled-up process. The results for the up-scaled purification are depicted in table 3.3.

Table 3.3: Purification table for cytochrome b5 isolated from 1500 mL ovine liver microsomes.

Fraction	Total Volume (mL)	[Protein] (mg/ml)	Cytochrome b_5 (µM)	Total Cytochrome b_5 (µmol)	Specific Activity (µmol/mg)	Fold Purification	Yield (%)
Triton X-100 filtrate	1500	4.19	1.78	2.68	0.0004	1.0	100
1st DEAE Eluate	290	1.40	9.12	2.64	0.0065	15.3	98.8
2nd DEAE Eluate	82	1.01	20.3	1.67	0.0201	47.2	62.2
3rd DEAE Eluate	66	1.03	22.3	1.47	0.0216	50.8	55.0

3.3.5 Gel electrophoresis and Western blot analysis of cytochrome b5

The purity of cytochrome b5 in isolated fractions was analysed using gel electrophoresis. The SDS-PAGE revealed that a protein with a molecular weight of *ca.*16 kDa could be observed, together with other high molecular weight proteins. The latter proteins had the same molecular weight as that of the protein with the most prominent band present in the microsomes (*ca.* 60 kDa). A duplicate gel was further subjected to Western blot analysis using rabbit anti-cytochrome b5 IgG. These antibodies recognised both the prominent bands, as well as the high molecular weight protein present in microsomes. These prominent bands represent the monomer (16 kDa) and the tetramer, which can be seen at approximately 60 kDa. Confirming previous findings of multimeric complex formation by cytochrome b5 *in vitro* [3].
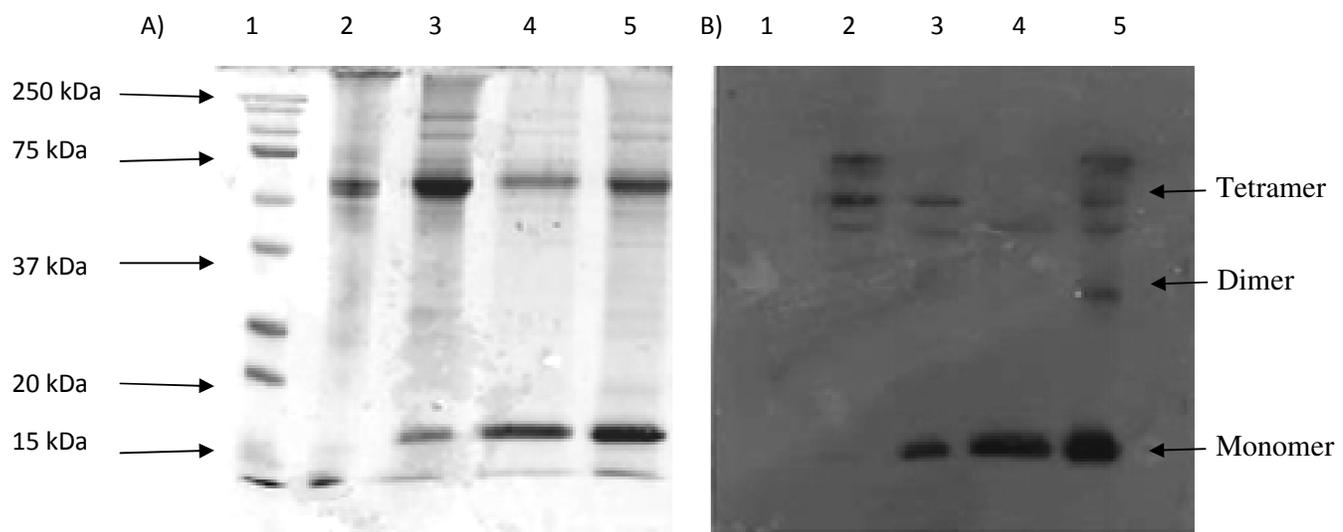
Figure 3.3: SDS-PAGE and Western blot analyses of purified cytochrome b5 fractions. For SDS-PAGE (A) and Western blot (B) lanes; 1: Kaleidoscope Molecular weight marker, 2: Solubilised microsomes (10 µg), 3: Cytochrome b5 from first column chromatography step (10 µg), 4: Cytochrome b5 from second column chromatography step (10 µg) and 5: Cytochrome b5 from third column chromatography step (10 µg). For the Western blot analyses a 1:1 000 primary antibody and a 1:20 000 secondary antibody dilution was used.

Furthermore, it is also of note that other complexes are visible on both the polyacrylamide gel and the Western blot. These complexes could possibly be regarded as dimers (30-32 kDa), trimers (45-48 kDa), pentamers (75-80 kDa), hexamers (90-96 kDa), etc. In addition, the latter multimeric complexes are not as prominent as the monomer (16 kDa) and the tetramer (60-64 kDa) in the purified fractions, but more imminent in the microsomal suspension. On

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the Western Blot the presence of a dimer can be observed. This dimer is not clearly visible on the polyacrylamide gel. This could be as a result of sensitivities between the techniques. Figure 3.3 shows the stained gel together with the accompanying immunoblot developed on an X-ray film.

3.3.6 Determination of Triton-X100 in dialysed fraction

The purification of cytochrome b5 from animal tissue involves a number of steps which contains the detergent Triton X-100. These steps include the solubilisation of membranes, to release all membrane bound proteins, and the first two chromatographic steps [4]. Although the third and final chromatographic step involves the removal of all detergents through extensive washing, it was found that traces of detergents could still be detected. When determining the accurate mass of proteins in solution, using ESI-MS, it is crucial to have the sample free of any detergents as Loo *et al.* [74] reported that in many cases the ion signals from surfactants dominate and obscure protein mass spectra resulting in inaccurate mass determination.

The absorbance spectra of each dilution were recorded and the fourth derivative spectra were calculated using the Cary Beckmann UV-100 software. The fourth derivative spectra for each was plotted (Figure 3.4) and the intensities of the spectral bands increased as the concentration increases.

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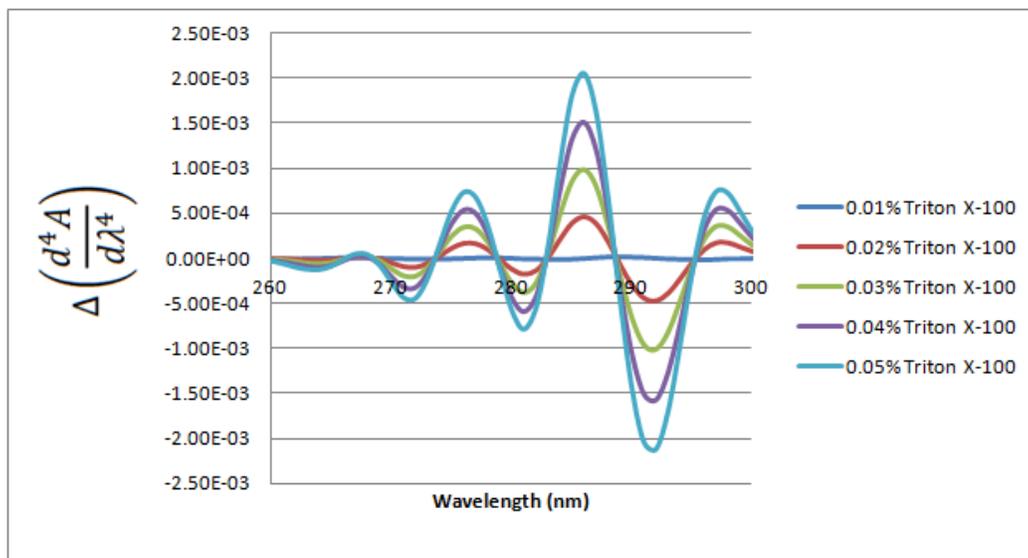
Figure 3.4: The fourth derivative spectra of Triton X-100. The increase in the percentages of Triton is shown within the figure, ranging from 0.01% Triton X-100 to 0.05% in 1 M KOH.

The spectral data from figure 3.4 was used to construct a linear standard curve for the determination of Triton X-100. This was done by taking the difference at the fourth derivative wavelengths, the apex (X) at 276 nm and the valley (Y) at 280 nm. By plotting these against their relative Triton X-100 concentrations, a standard curve (figure 3.5) was obtained and used to determine the Triton X-100 content of the cytochrome b5 dialysate to be less than 0.004%.
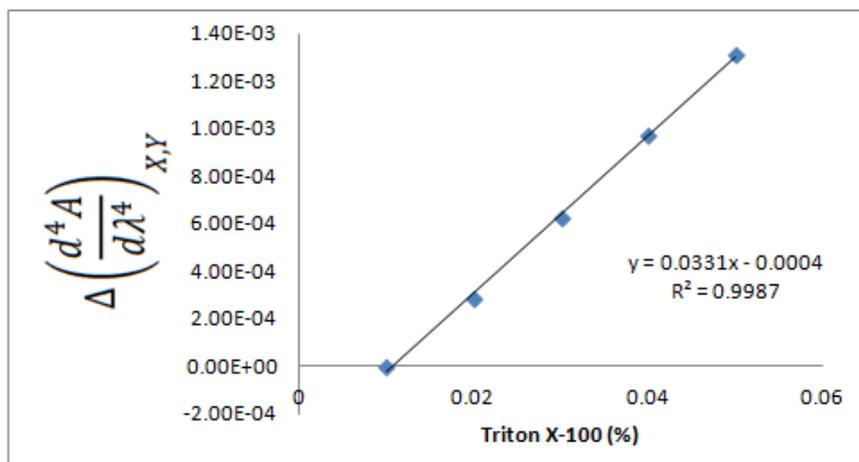
Figure 3.5: Standard curve obtained from the difference at wavelengths X and Y, 276 nm and 280 nm respectively. The amount of Triton X-100 present in protein solution was determined, from the standard curve, as 0.004%.

3.3.7 Accurate mass determination of purified cytochrome b5

Although it was found that there was less than 0.01% Triton present in the purified fraction after dialyses, this trace amount of detergent was enough to obscure the results obtained from ESI-MS. When cleaning the sample using a detergent removal column, it could be seen that ESI-MS could only detect a single heme containing protein and no other contaminants, such as detergents (figure 3.6). The molecular ions detected using mass spectrometry were not only that of the protein, but also the heme group. The heme can be seen on the ESI-MS spectrum with an accurate molecular mass of 616 Dalton. Deconvolution of the ESI-MS spectrum, figure 3.7, calculated the accurate monoisotopic mass of the detected protein to be 15249 Da (theoretical M_r = 15330 Da), which correlated with the molecular weight previously reported by Lombard *et al.* [3]. According to the ESI-MS analysis the M_r of intact cytochrome b5 would therefore be 15865 Da, correlating with the 16 kDa band we observed for the monomeric cytochrome b5.

Figure 3.6: Multiprotonated ESI-MS positive-mode spectrum of the isolated cytochrome b5 fraction. A single protein which contains a protoporphyrin IX heme group with an accurate molecular weight of 616 Da was detected.
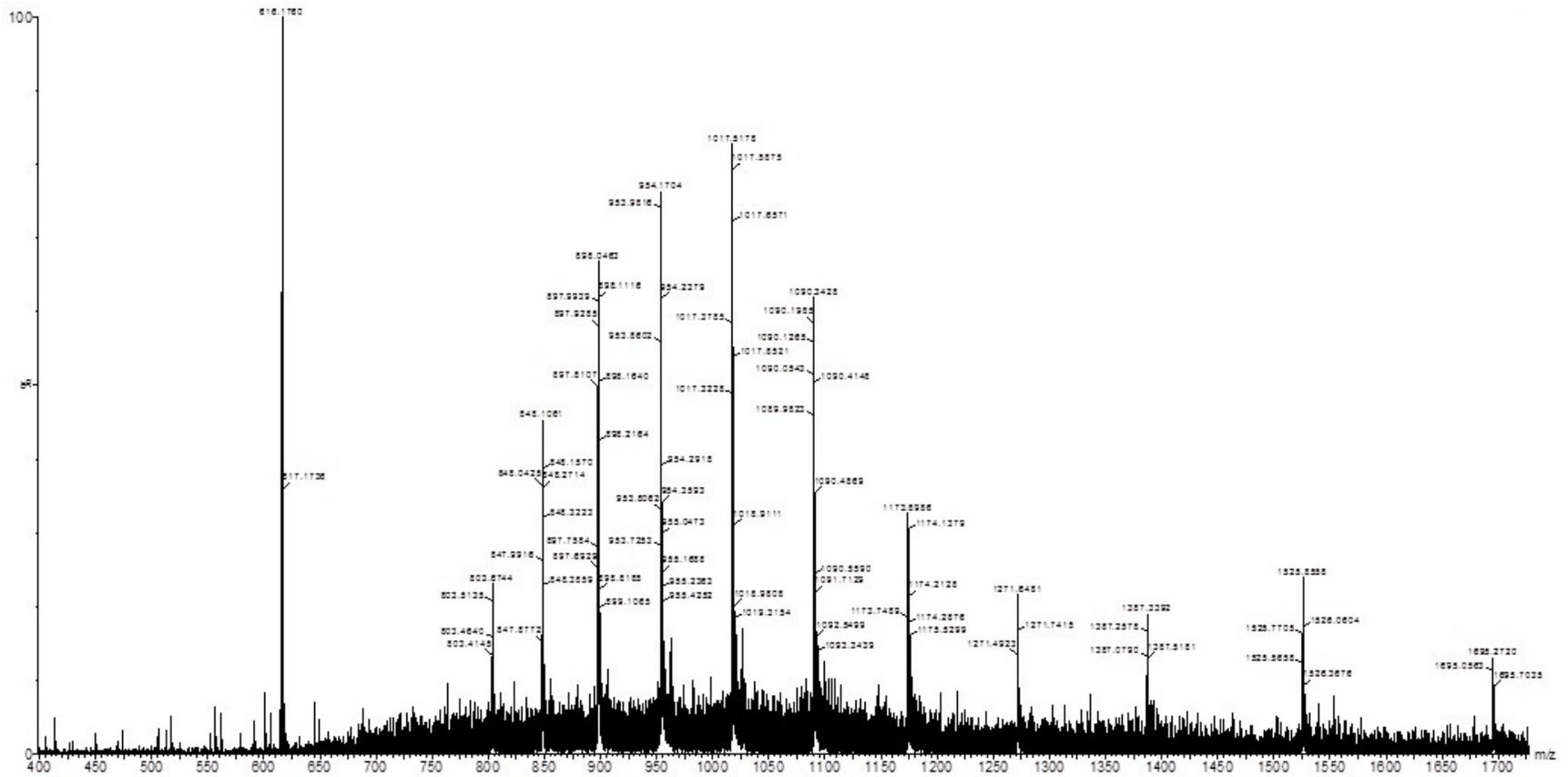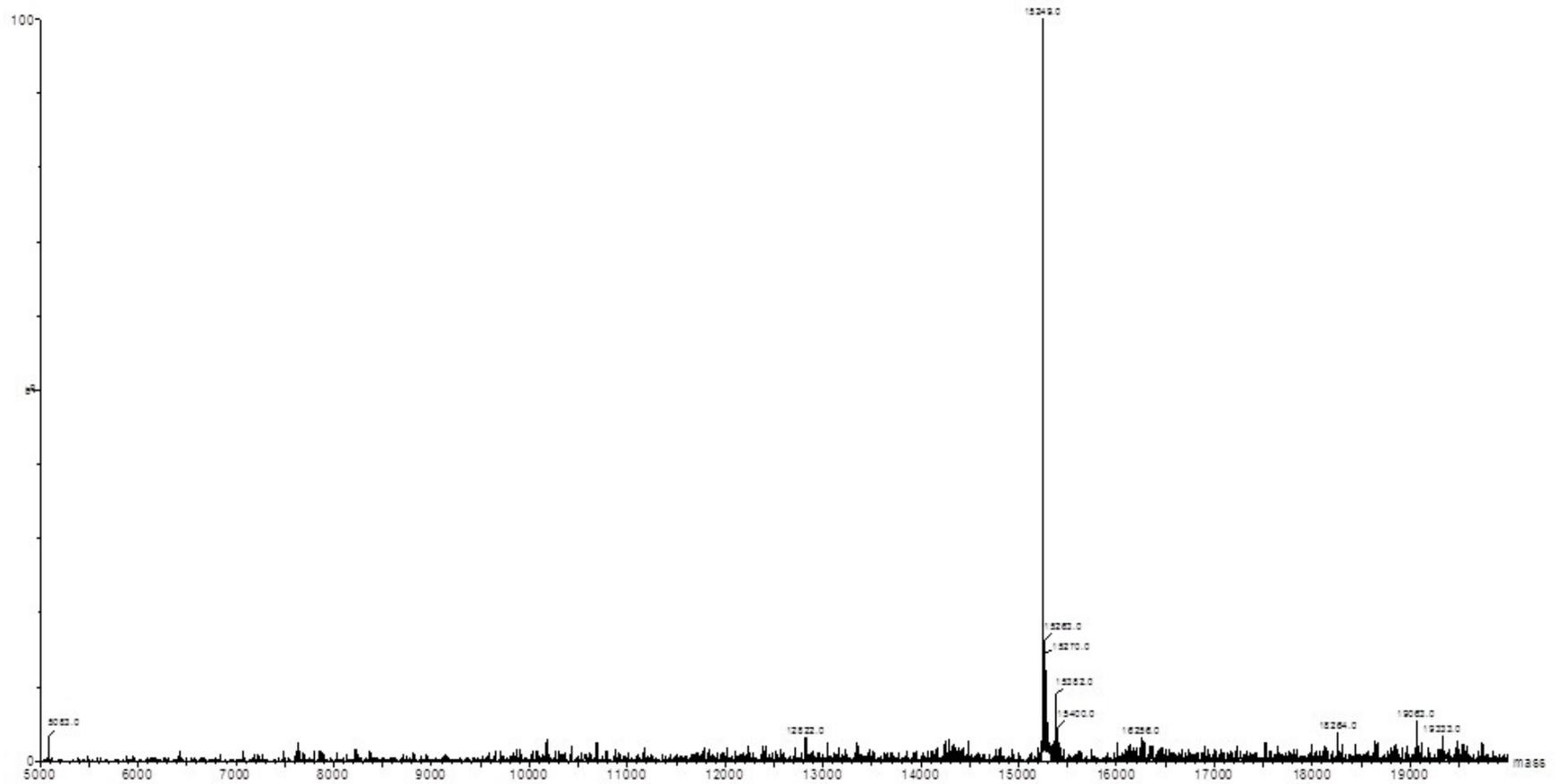
Figure 3.7: Deconvoluted spectrum of the multiprotonated spectrum of the cytochrome b5 preparation. The molecular mass of wild-type cytochrome b5, without heme, was determined to 15 249.0 Da.

3.4 Conclusion

Cytochrome b5 was previously isolated from ovine liver microsomes using the PEG fractionation method [3]. In this chapter, employing this method cytochrome b5 was successfully isolated from animal tissue.

Spectral analyses showed that homogenisation steps did not affect the activity of the protein (figure 3.1) as cytochrome b5 could still be reduced by the cytochrome b5 reductase present in the microsomal preparation. Furthermore, solubilising the membranes using a mild detergent, Triton X-100, followed by the subsequent purification with multiple chromatographic steps showed cytochrome b5 was still capable of being reduced with sodium dithionite in the absence of its redox partner (figure 3.2).

Purification data revealed cytochrome b5 was purified from ovine liver microsomes up to 120 fold with less than 50% loss in the purification steps. During this study, cytochrome b5 was successfully purified a number of times. We also found that during up-scaling the purification, no significant difference in the yield could be noticed but a considerable decrease in the fold purification was observed. This can be seen by comparing tables 3.2 and 3.3. From these data it could be concluded that when purifying more than 1 L of solubilised microsomes the third and final chromatographic step could be omitted, as this step did not lead to a significant increase in the fold purification but has the same loss (about 50%) in terms of yield as the "normal" scale.

SDS-PAGE and Western blot analysis confirmed the presence of the full length protein, with most prominent bands visible at ~16 kDa and ~60 kDa. Other multimeric forms could also be observed, such as dimers, trimers and hexamers, but were not as prominent as the monomer and tetramer. Previously it has been reported that cytochrome b5, isolated from animal tissue, forms multimeric complexes [3][6]. These bands could also be detected using anti-cytochrome b5 immunoglobulins, further confirming the presence of cytochrome b5 (figure 3.3).

Determining the amount of detergent left in the protein showed that there was less than 0.01% Triton X-100 left after dialysis, this trace amount was enough to interfere with the MS signal. Using detergent removal columns, to totally remove the trace amount of detergent left, yielded ESI-MS spectrum (figure 3.6) with minimal background in the preparations. ESI-MS showed a single, heme containing protein with an accurate *apo*-protein mass of 15 249 Da

and the heme group to be 616 Da (figure 3.7). The molecular weight of cytochrome b5, determined by ESI-MS, correlated with the molecular mass of ovine *apo*-cytochrome b5 determined from its amino acid sequence as 15330 Da.

In Chapter 4 the investigation of the multimeric complexes of cytochrome b5 will be described. The appearance of multiple protein bands on SDS-PAGE, observed during purification of cytochrome b5, was investigated using different denaturing conditions as well as protein aggregation using a fluorescent dye. A number of techniques were employed to investigate the aggregation of the protein and factors which influence this aggregation.

Chapter 4

4. Investigating aggregate formation of Cytochrome b5

4.1 Introduction

The molecular mass of ovine cytochrome b5 (without the heme) was previously determined by ESI-MS in our laboratory and was reported to be 15263 Da [3][6]. Also, in Chapter 3 we have reported the accurate molecular mass of isolated cytochrome b5 as 15249 Da, without the heme. The ESI-MS analyses from these studies showed that the cytochrome b5 preparation contained a single monomeric heme containing protein.

Previous studies in our laboratory have reported cytochrome b5 from animal tissue forms SDS stable multimeric complexes as visualised by SDS-PAGE. The cytochrome b5 that was isolated in this study (Chapter 3) showed similar behaviour. These aggregation states have, however, only been identified *in vitro*. Lombard *et al.* [3] reported that during separation of the full length cytochrome b5 protein by SDS gel electrophoresis, multiple protein bands could be observed. By removing the membrane anchoring domain through tryptic digestion, it was shown that the aggregation of the protein was totally abolished [3]. More recently, it was shown that aggregate formation by cytochrome b5 was not due to an artefact of the isolation procedure, but also occurred *in vivo*. Storbeck *et al.* [6] showed that heterologously expressed cytochrome b5 forms homomeric complexes in a COS-1 cell line. Furthermore, our studies supported the hypothesis that the membrane binding domain plays a pivotal role in the aggregation.

In this chapter a broader investigation of the multimeric complex formation and aggregation of cytochrome b5 is described. As mentioned earlier, purified cytochrome b5 yields multiple protein bands when subjected to SDS-PAGE analyses and the most prominent protein bands are visible at molecular masses corresponding to 15 kDa and 60 kDa respectively. These bands were subsequently excised and identified using nano-LC-MS/MS. The aggregation of the protein was also investigated at different protein concentrations and ionic strengths. The role of the membrane binding of cytochrom b5 domain was also elucidated.

4.2 Experimental Procedures

4.2.1 SDS gel electrophoresis under extensive denaturing conditions

Conventionally, sample preparation for denaturing gel electrophoresis involves the addition of Laemmli® [72] sample buffer to the protein sample in a 1:1 ratio followed by a heat treatment step. To analyse the aggregation of cytochrome b5, a number of different denaturing conditions were investigated prior to analyses on a 12.5% SDS-PAGE as described in *section 3.2*. Protein samples were prepared, after the addition of Laemmli® sample buffer, through: boiling for 10 minutes, boiling for 10 minutes in the presence of 8 M urea, incubation at 40°C for 30 minutes and incubation at 40°C for 20 minutes in the presence of 8 M urea.

4.2.2 Isolation of immunoglobulins specific for cytochrome b5 aggregates

Western blot analyses of purified cytochrome b5, using polyclonal antibodies, yielded five prominent immuno-precipitation bands corresponding to the monomeric form of cytochrome b5 at 16 kDa, dimeric form at 32 kDa and a tetrameric form corresponding to a molecular mass of 60 kDa. Other forms, which were not identified but recognised by immunoglobulins, were the trimeric form at 48 kDa and a pentameric form at 80 kDa (Figure 3.3). We attempted the purification of the antibodies using the monomer and the most prominent aggregate, the tetramer, as immuno-affinity ligands to ascertain if the monomer presented the same antigenic determinants as the tetramer as obtaining antibodies specific for either form would be invaluable in the investigation of cytochrome b5 complex formation *in vivo*. A 12.5% SDS gel was subsequently used to separate cytochrome b5 under the conditions previously described (*section 3.2.8*). The separated proteins were transferred to a nitrocellulose membrane (BioTrace™ NT – Life Sciences) where after it was stained with Ponceau S Solution to visualise the transferred proteins. Two membrane strips, containing the proteins bands with the most prominent signal, at 16 and 60 kDa respectively, were cut out and extensively washed with water prepared by reverse osmosis (RO-water) to remove all Ponceau S solution. After removal of staining solution, the strips were incubated for 30 minutes in casein blocking buffer. The strip, corresponding to 60 kDa, was subsequently incubated for 90 minutes in primary antibody solution (1: 500). Immobilised immunoglobulins were removed by incubating the strip in 5 mL of a 0.2 M glycine, pH 2.5 for 4 minutes followed by transfer to 0.5 mL 1 M TRIS-HCl buffer, pH 8.0, and the pH was

adjusted to 7.0 using NaOH. This step was repeated 3 more times using the same primary antibody solution. After the third immunoglobulin isolation step, the same serum solution (used for the isolation of the 60 kDa antibody) was used and the procedure described above was repeated for the 16 kDa strip. These affinity purified immunoglobulins were used, undiluted, for Western blot analyses (as described in *section 3.2.9*) to assess if the antibodies isolated were specific for either the monomer or the aggregate [6]. The visualisation method for the western blot differed for the the method described in the previous section. For this NBT/BCIP solution was used for colour development (at least 2 minute exposure).

4.2.3 SDS-PAGE of cytochrome b5 and excision from SDS gel

Mass spectrometry based proteomics was performed to unambiguously identify the bands present on SDS polyacrylamide gel as cytochrome b5. Cytochrome b5 was subjected to SDS-PAGE analyses as previously described. The gel was stained using Coomassie stain for 45 minutes and destained overnight. Further analyses, as described in this section, were performed by the Central Analytical Facility of the University of Stellenbosch. Bands, corresponding to 16 and 65 kDa respectively, were excised from the gel and cut into smaller squares, approximately 1 mm × 1 mm. Each band was transferred to a separate 1.5 mL Eppendorf, where it was first washed with 2 × 300 µL of deionised water followed by washing with a 50% (*v/v*) acetonitrile in water. After 10 minutes the acetonitrile mixture was replaced with 50 mM ammonium bicarbonate and incubated for 10 minutes. The acetonitrile and ammonium bicarbonate washing steps were repeated until all the Coomassie stain was completely removed, usually 3 – 4 wash steps. The final wash step was followed by incubating the gel squares in acetonitrile until a white discolouration occurred, the acetonitrile was removed and the gel was dried in a vacuum.

4.2.4 Reduction, alkylation and trypsin digestion of intact protein

Although sample preparation for the gel electrophoresis involves the reduction of disulfide bonds, it is necessary to treat the separated proteins with a reducing agent followed by the irreversible alkylation of the thiol groups preventing the formation of disulfide bonds. The dried gel squares were incubated for 60 minutes at 57°C with a reducing agent (10 mM DTT, 100 µL) where after it was washed once as previously mentioned (*section 4.2.3*). The alkylation of the proteins was subsequently accomplished by incubating the gel squares with 55 mM iodoacetamide (100 µL) for 60 minutes in the dark, followed by washing with a 50 mM ammonium bicarbonate solution for 10 minutes followed by 50% (*v/v*) acetonitrile/water

for 20 minutes before drying under vacuum. Digestion of the proteins was accomplished by the addition of 200 ng of trypsin in solution and incubating for 17 hours at 37°C. The resulting peptides were isolated from the gel by separating the trypsin from the gel squares and extracting the peptides with 2 x 300 μl 70% (*v/v*) acetonitrile in 0.1% (*v/v*) formic acid in water for 30 minutes.

4.2.5 Removal of salts and other contaminants from extracted peptides

To decrease the interference on MS by adducts the salts were removed from each sample. The solution containing the isolated peptides was subjected to a stage tip, containing C_{18} matrix, to remove all salts. The C_{18} matrix was activated by washing with 300 μL methanol. The stage tip was equilibrated with 300 μL of 0.1% formic acid, where after the sample was applied. The C_{18} material was washed with 2×300 μL of H_2O and the peptides were eluted with 2×300 μL 70% (*v/v*) acetonitrile in 0.1% (*v/v*) formic acid. Peptides were dried and stored at −20°C until analyses.

4.2.6 Nano-Liquid Chromatography of peptides

Mass spectrometry was performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos MS (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray source. Separation of the peptides was achieved chromatographically, using a two column chromatographic step on an EASY-Column (2 cm, ID 100 μm, $C_{18,}$ 5 μm particle size) pre-column followed by an EASY-Column (10 cm, ID 75 μm, $C_{18,}$ 3 μm particle size), at a flow rate of 300 nL/min. The peptides were dissolved in 20 μL 20% (*v/v*) acetonitrile containing 0.1% (*v/v*) formic acid and 10 μL was injected. The chromatographic conditions were as follows: Solvent A; H_2O containing 0.1% (*v/v*) formic acid, Solvent B; 100 % (*v/v*) Acetonitrile containing 0.1% (*v/v*) formic acid and a gradient from 5−40% solvent B over 20 minutes, 40−80% solvent B over 5 minutes and kept at 80% solvent B for a further 10 minutes to complete the run.

4.2.6 MS/MS analyses of peptides

The mass spectrometer was operated in data dependent mode. This allowed for automatic switching between the acquisition of the Orbitrap-MS and the LTQ-MS/MS. The former acquires the precursor ion spectra, with resolution R = 60 000. Further fragmentation took place in the linear ion-trap using an induced dissociation. Conditions for the mass

spectrometer were 1.5 kV, 200°C with no sheath and auxiliary gas flow and ion selection threshold of 500 counts for MS/MS.

4.2.7 Investigation of cytochrome b5 aggregation using a fluorescent dye

1-Anilinonaphthalene-8-sulfonic acid (ANS) is a fluorescent dye commonly used in the study of protein aggregation in solution. The molecule is an amphiphilic probe of which the fluorescence increases when it enters a hydrophobic environment. As it was previously reported that the membrane anchoring domain plays a vital role in the aggregation of the protein [6], we used ANS to investigate whether the aggregation could cause an increase in ANS fluorescence.

4.2.7.1 Effect of protein content on aggregation

To study the effects on protein concentration, cytochrome b5 (100 µg; final volume 150 µL) a dilution series was prepared in a 96 well NUNC^{TM} fluorescence plate with PBS as diluent. The loaded plate was covered and pre-incubated at 37°C and after 20 minutes, 1.11 µL of 6.5 mM ANS (50 µM final concentration) was added to each well and the 96- plate was incubated at ambient temperature for another 15 minutes. This was considered sufficient time for the interactions to fully proceed, where after the fluorescence was measured using a Thermo Varioskan fluorescence detector equipped with Varioskan software. The excitation wavelength was set to 388 nm and each well was scanned for fluorescence emission over a wavelength range ranging from 420 to 600 nm. Cytochrome C, another heme containing protein, was used as a control protein for the experiment. Wells containing only the protein solution, without any fluorescent probe, were also included as negative controls.

4.2.7.2 The effect of ionic strength on cytochrome b5 aggregation

A number of factors have been shown to have an effect on the aggregation state of protein complexes, including ionic strength. To study this effect on cytochrome b5 complex formation, cytochrome b5 (1.1 mg/mL) was diluted, using phosphate buffer having different molar concentrations ranging from 5 mM – 1 000 mM, to a final protein concentration of 0.1 mg/mL. These dilutions were pre-incubated overnight at 4°C, where after it was transferred to a NUNC^{TM} fluorescence plate (200 µL/well) and incubated at 37°C. After 20 minutes ANS was added, the mixture incubated for 15 minutes and the fluorescence measured as described in *section 4.2.7.1.*

4.2.7.3 The effect of the membrane binding domain of cytochrome b5 on aggregation

Previous studies in our laboratory showed that, when removing the membrane binding domain of cytochrome b5 through tryptic digestion, a protein with a single molecular mass of *ca*. 10 kDa, was observed [3]. This form of the protein was named truncated cytochrome b5.

Truncated cytochrome b5 was prepared as described by Lombard *et al*. [3]. Briefly, 1 mg of cytochrome b5 was immobilised on a DEAE-cellulose column, dimensions 1×5 cm, equilibrated with 10 mM TRIS-Acetate buffer, pH 8.1 containing 25 mM $CaCl_2$. After protein immobilisation, the column was washed with 20 mL equilibration buffer followed by the circulation of trypsin solution. Trypsin at 5000 units/mL was continuously circulated through the column at 4°C. After 16 hours, the column was extensively washed, 10 column volumes, with equilibration buffer to remove all traces of resulting peptides and trypsin before the truncated cytochrome b5 was eluted using a 10 mM TRIS-Acetate buffer, pH 8.1, 250 mM Sodium Thiocyanate (NaSCN) containing 1 mM EDTA. The eluate was concentrated using a LABCONCO® CentriVap Concentrator followed by extensive dialysis against 10 mM TRIS-acetate buffer, pH 8.1 containing 1 mM EDTA. The total protein of the dialysate was determined using the BCA method described in *section 3.2.2* and analysed through SDS- PAGE as described by *section 3.2.8*.

The aggregation of the truncated form of the protein was analysed in the same manner as described earlier in *section 4.2.7.1*. Cytochrome b5 and truncated cytochrome b5 (100 µg) were diluted in series and incubated at 37°C for 20 minutes, where after ANS was added and the fluorescence was measured.

4.3 Results

4.3.1 SDS-PAGE under extensive denaturing conditions

Evidence from ESI-MS spectra previously obtained (*section 3.3.7*) indicated that the purified fraction was a single heme containing protein. Conversely, the SDS polyacrylamide gel indicated that the purified fraction does not contain a single protein band at the expected molecular weight of 16 kDa, but rather multiple protein species, with the most prominent bands corresponding to molecular masses of 16 and 60 kDa. Although these multi-species were observed, only a single protein could be detected by ESI-MS analysis (Chapter 3) which led us to the following conclusions; that these species might be homomeric complexes of

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cytochrome b5 and/or be a result of protein degradation or protein mixtures. The effect of different denaturing conditions was investigated and it was found that under different denaturing conditions the different aggregates persisted. As shown in Figure 4.1, no notable difference, either in lane profiles or in band intensities, could be observed regardless of the denaturing protocol followed. Furthermore, in lane 2 a human protein, phosphopantothenoyl cysteine decarboxylase, was added as a control. In its native form the protein exists in an aggregated state (usually 75 kDa) but completely denatures when separated on a SDS gel [76] and using the traditional method of protein denaturing, prior to electrophoresis, we were able to completely denature the protein to its monomeric form (25 kDa) .
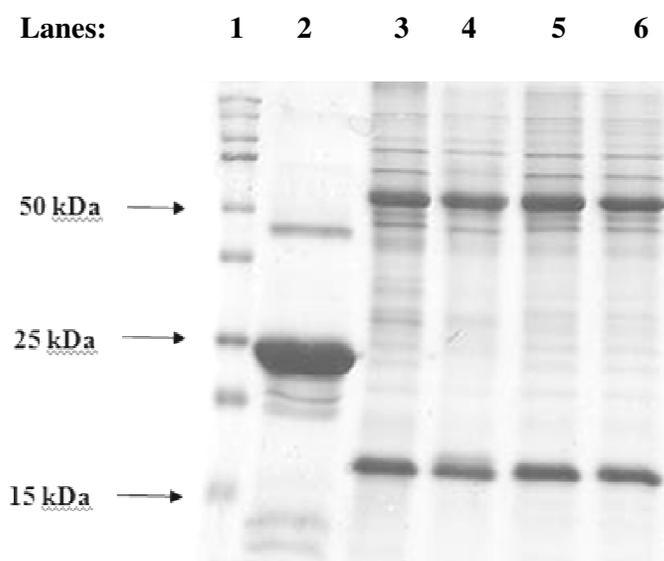
Figure 4.1: SDS polyacrylamide gel containing cytochrome b5 with different denaturing conditions, after the addition of equal volume Leammli® Sample Buffer. Lane 1: BioRad Precision Plus Protein Standard, lane 2: human protein phosphopantothenoyl cysteine decarboxylase (12 µg) boiled for 10 minutes, lane 3: cytochrome b5 (12 µg) boiled for 10 minutes, Lane 4: cytochrome b5 (12 µg) incubated for 30 minutes at 40°C, lane 5: cytochrome b5 (12 µg) containing 8 M urea, boiled for 10 minutes and lane 6: cytochrome b5 (12 µg) containing 8 M urea, incubated for 20 minutes at 40°C.

4.3.2 Isolation of immunoglobulins and reblotting for specificity

As shown on the immuno-blot, in Chapter 3 (Figure 3.3), antibodies raised against purified cytochrome b5 detected both the monomeric and tetrameric forms of the protein. These immunoglobulins were purified using an immuno-affinity method which involved the binding of the immunoglobulins to their antigens and their subsequent removal from solution through

incubation in a 0.2 M glycine solution at an acidic pH. When reused for Western blotting purposes, the immunoglobulins isolated from the tetrameric cytochrome b5 only recognised the tetrameric form of the protein and not the 16 kDa monomer. In contrast the monomeric antibody recognised both the monomer and the tetramer as antigen. The latter yielded a more intense signal indicating that the immunoglobulin recognising cytochrome b5 monomers showed more affinity for the tetrameric form rather than the monomeric form (figure 4.2).
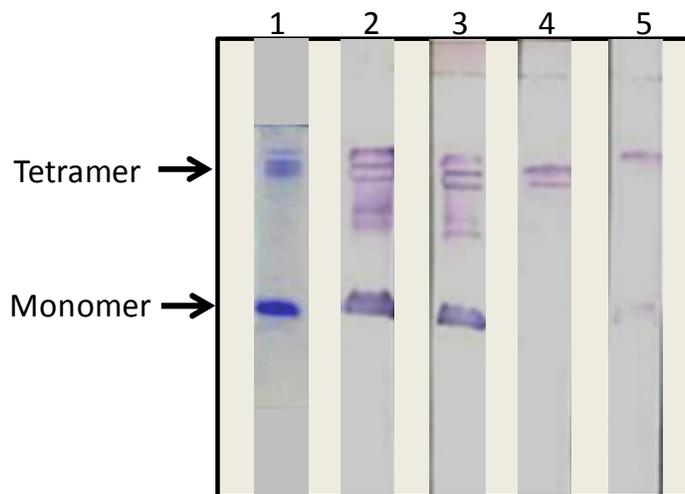
Figure 4.2: Western blot using affinity purified immunoglobulins. Western blot was completed with sera dilutions for strips 1 and 2, 1: 1000, and undiluted affinity serum (strips 3 and 4). Secondary antibody (goat anti-rabbit conjugated to alkaline phosphatase) 1: 20 000. Strip 1: stained SDS gel with cytochrome b5 (7 µg), strip 2 cytochrome b5 (7 µg) blotted with whole serum, strip 3: cytochrome b5 (7 µg) blotted with "stripped" serum, strip 4: cytochrome b5 (7 µg) blotted with tetramer immunoglobulins and strip 5: cytochrome b5 (7 µg) blotted with monomer immunoglobulins.

4.3.3 Identification of excised bands using nLC-MS/MS

As both the 16 and 60 kDa bands were indicated to be cytochrome b5, through recognition by affinity purified immunoglobulins, we further investigated these bands using nLC-MS/MS. This process involved the identification of peptide amino acid sequences and comparing it against a library. Two bands, the monomer (16 kDa) and tetramer (60 kDa), from the SDS-PAGE gel were excised and further analysed for the presence of cytochrome b5. The excised bands underwent proteolytic digestion, using trypsin, and the resulting peptides were further reduced and alkylated, where after it was separated using nano-liquid chromatography before ionisation. MS/MS analyses on the generated peptides confirmed that their amino acid sequences, when compared with library sequences, originated from cytochrome b5 as

indicated by high scores and coverage (table 4.1). Cytochrome b5 was therefore present in both bands detected with SDS-PAGE.

Table 4.1: Identification of excised bands with nLC-MS/MS

Molecular Weight on SDS Gel (kDa)	Sequence Matched (Species)	Score	Coverage	No. of peptides	No. of Amino Acids	Molecular Weight (kDa)	Calculated pI
16	Cyt b5 from Bovine	37.78	31.34	3	134	15.3	5.03
60	Cyt b5 from Ovine	8930.03	59.7	10	134	15.3	5.16

4.3.4 Investigating aggregate formation

4.3.4.1 Effect of protein concentration on aggregation

The effect of protein concentration on the aggregation has been previously investigated by the Adriaanse [77]. It was found that the aggregation of cytochrome b5 could be manipulated by the concentration. Through serial dilution of cytochrome b5, it was shown that the presence of the tetramer decreased while that of the monomer remained (figure 4.3). Analysing the densities of the most prominent protein bands, which represents the monomer, trimer and tetramer, it was concluded that the aggregation was, indeed, concentration dependent.
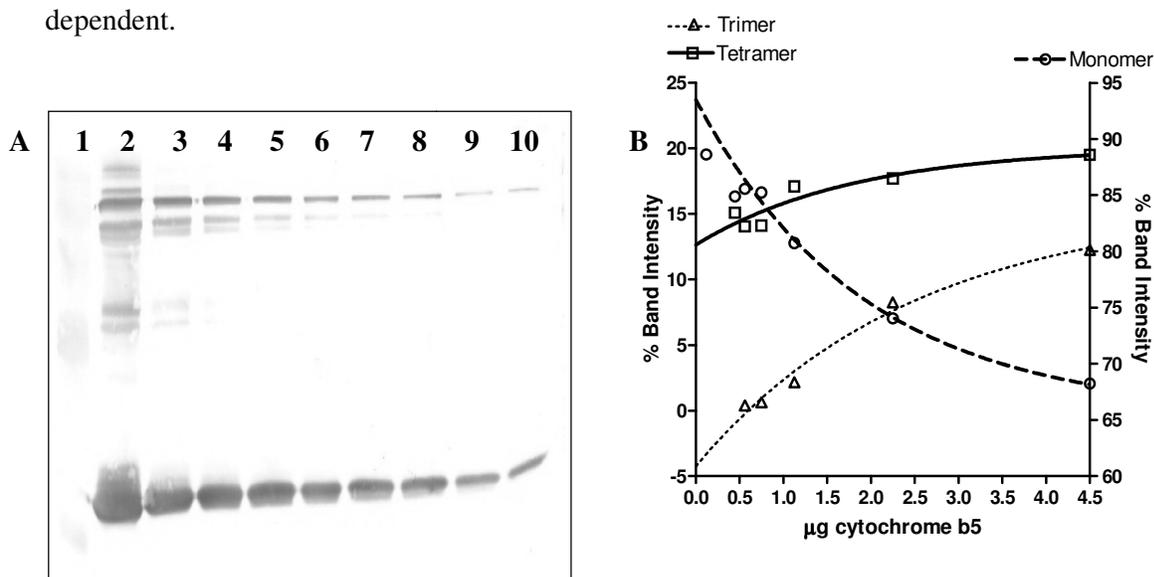
Figure 4.3: Previous studies [77] showing the effect of protein concentration on the aggregation of cytochrome b5. The SDS-PAGE gel is shown in A with lane 1 containing the molecular mass marker (3 µg), lanes 2 – 10 dilution of cytochrome b5 ranging from 22 µg to 0.13 µg. A graphical representation of the concentration dependence of cytochrome b5 aggregation as detected by SDS-PAGE is given in B.

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For the new studies, using the fluorescent dye ANS to study cytochrome b5, we included a number of controls, one of which was cytochrome C, a small heme containing protein, which is also involved in electron transfer reactions and has a similar molecular mass as that of cytochrome b5. ANS is commonly used for studying peptides and proteins which form complexes. When proteins or peptides aggregate, they form hydrophobic crevices. When the ANS is present it occupies these hydrophobic pockets and when excited it emits fluorescent light with maximum at 470 nm. The fluorescence detected is also directly proportional to the amount of aggregation present within the solution.

As ANS's fluorescence increases when embedded within a hydrophobic crevice, it was important to include cytochrome C as control because both cytochrome C and cytochrome b5 contain heme groups which are located in a hydrophobic pocket. Thus, the only difference between these proteins is the fact that we found that cytochrome b5 forms SDS stable aggregates and cytochrome C does not.

ANS fluorescence experiments confirmed that cytochrome b5 forms complexes or higher aggregates that is concentration dependent (figure 4.4). The increase of ANS fluorescence was significant when the ANS and the purified protein were present in the same environment. A serial dilution of cytochrome b5 caused a decrease in the fluorescence, while varying the cytochrome C concentration (figure 4.5) did not have any effect on the fluorescence. This suggested that cytochrome b5 forms aggregates *in vitro* and that the aggregation is dependent on the amount of protein present, with onset of aggregation at 0.95 µg/ml and 50% aggregation calculated at 1.56 µg/mL (figure 4.6). These observations correlated very well with our SDS-PAGE results from a previous study [77] (figure 4.3). Recently our group has also shown that the *in vivo* aggregation state of cytochrome b5 was dependent on the amount of expressed protein, and this study also showed a definite decrease in fluorescence as the amount of protein decreased [6].
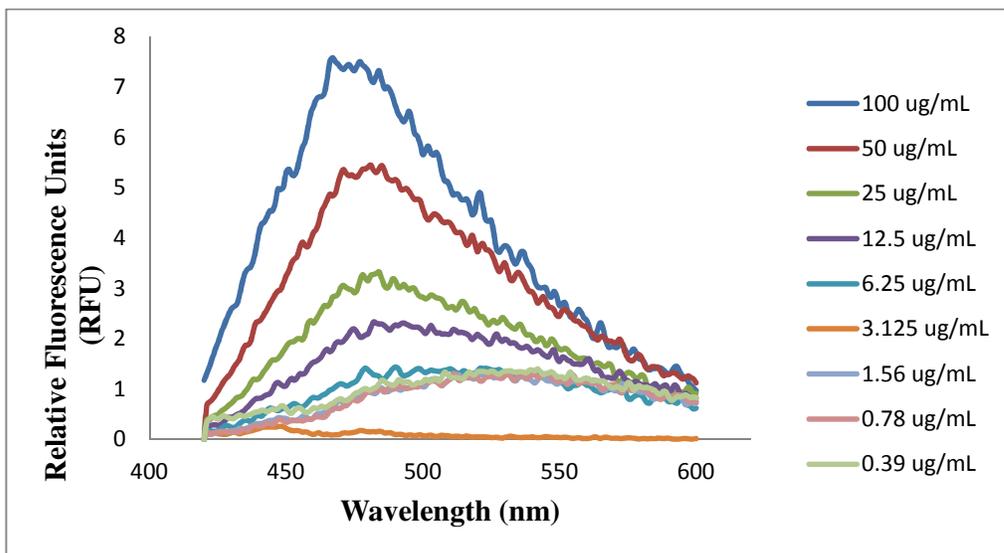
Figure 4.4: Emission spectra of 50 µM ANS in the presence of different concentrations of purified full length cytochrome b5 in PBS.
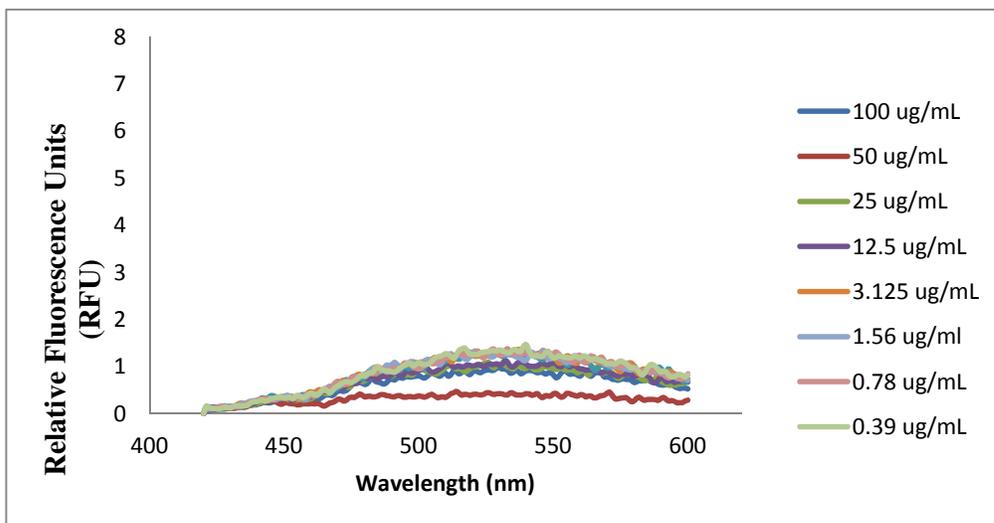
Figure 4.5: Emission spectra of 50 µM ANS in the presence of different concentrations of cytochrome C in PBS
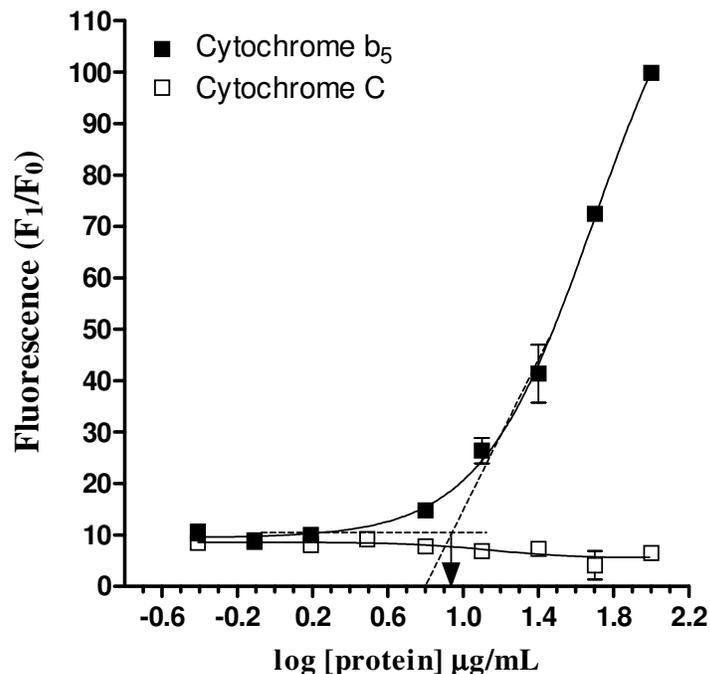
Figure 4.6: ANS fluorescence at 470 nm as a function of protein concentration. The arrow indicates the onset of aggregation at 0.95 µg/mL. The protein concentration at 50% aggregation was determined to be 1.56 µg/mL. The data points depicted are the mean ± standard error of two determinations.

4.3.4.2 Effect of ionic strength on protein aggregation

Lombard *et al.* [3] previously reported that cytochrome b5's aggregation/complex formation was affected by the ionic strength of the buffer. We investigated this by pre-incubating the same amount of protein in a phosphate buffer with an increase in ionic strength. For this, 100 µg of cytochrome b5 was incubated in phosphate buffer, pH 7.2, ranging from 5 mM to 1000 mM in concentration. It was confirmed that the ionic strength had an effect on the aggregation (figure 4.7). As the ionic strength increased, there was an increase in the fluorescence indicating increased cytochrome b5 aggregation.
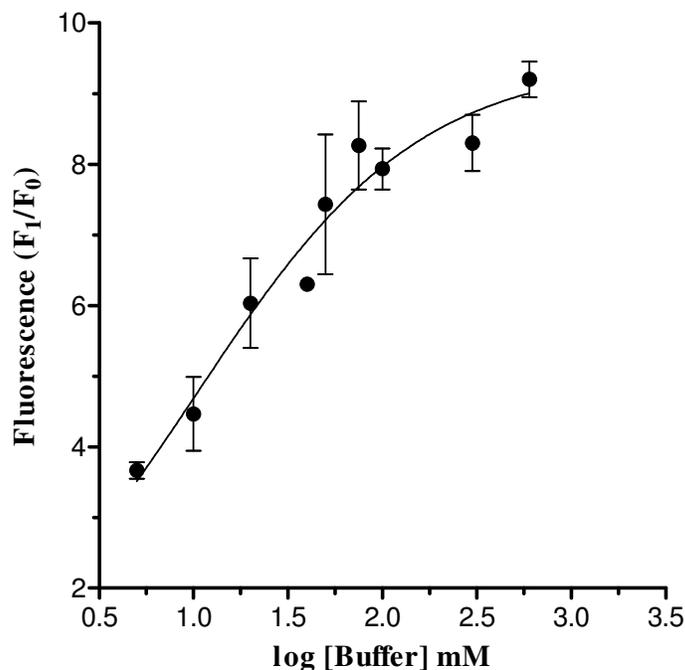
Figure 4.7: Effect of ionic strength on cytochrome b5 aggregation. This was generated with a non-linear fit to three analyses repeats.

4.3.4.3 Effect of the membrane binding domain on aggregation

More recently, studies by our group indicated that the membrane anchoring domain plays a vital role in the aggregation of cytochrome b5 [3][6]. We also investigated this with fluorescence using the truncated form of the protein. Truncated cytochrome b5 was prepared using the method described by Lombard [5]. This involved the overnight exposure of full-length cytochrome b5 to trypsin. After 16 hours of digestion, the truncated form of the protein eluted from the column as a distinct sharp peak. This peak was collected, dialysed, concentrated and the protein concentration was determined to be *ca.* 1.6 mg/mL.

The purity of the fraction was determined with SDS gel electrophoresis (figure 4.8 A) and it showed that the truncated form of the protein was present (11 kDa) with a slight contamination of trypsin (faint band at 25 kDa). In contrast to the full length cytochrome b5, no increase in ANS fluorescence was detected for the truncated form of the protein upon an increase in protein concentration (figure 4.8 B). This finding confirmed that the truncated form did not form aggregates and that the membrane binding domain plays a pivotal role in cytochrome b5 complex formation.
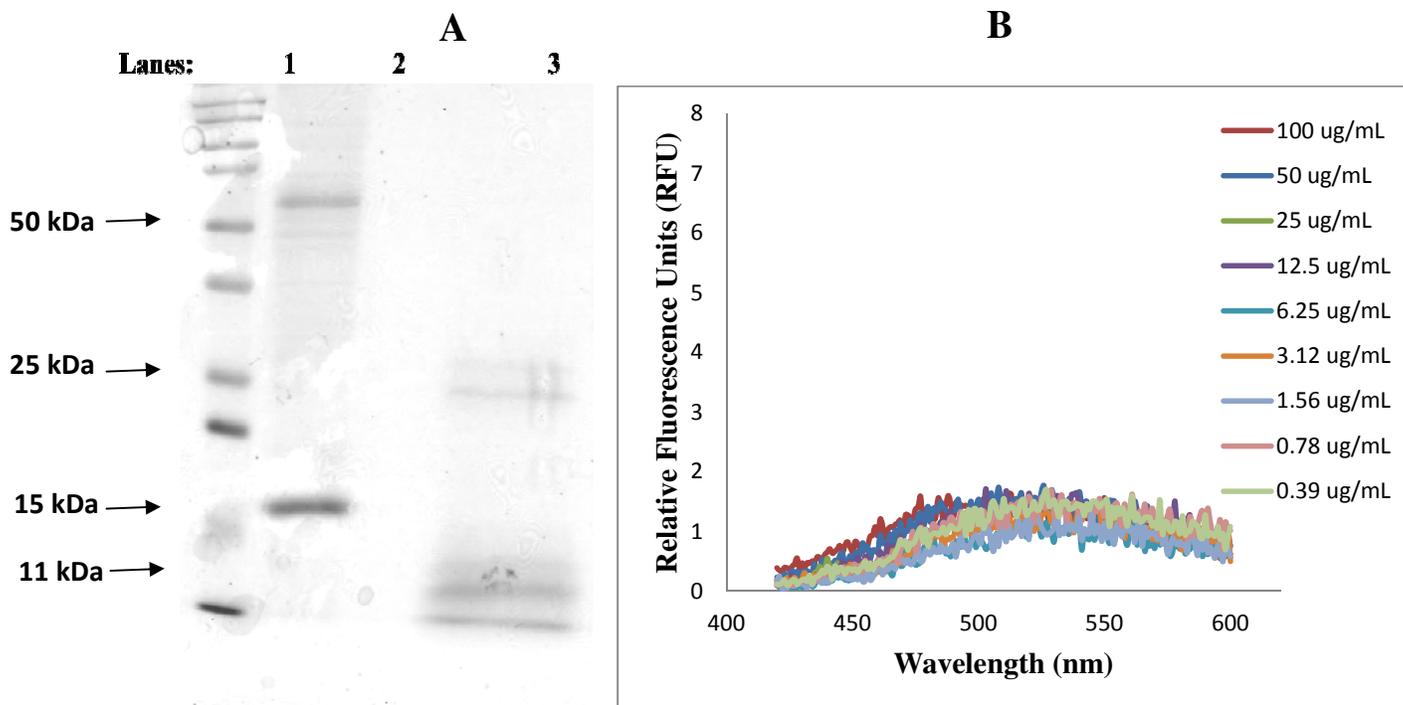
Figure 4.8: SDS polyacrylamide gel and ANS fluorescence for truncated cytochrome b5 A) SDS gel containing in lane 1: BioRad Precision Plus Protein Standard, lane 2: full length cytochrome b5 (5 µg) and lane 3: truncated cytochrome b5 (5 µg). B) Serial dilution of truncated cytochrome b5 from 100 µg/mL – 0.39µg/mL.

4.4 Conclusion

The molecular mass of cytochrome b5 (without heme) has been previously reported to be 15.2 kDa and SDS-PAGE analyses of the purified cytochrome resulted in multiple protein bands [3][6], correlating with our studies. For both the studies of Lombard *et al.* [3] and Storbeck [6], ESI-MS analysis indicated only one heme containing monomeric protein in the purified protein preparation with the molecular mass correlating with that of cytochrome b5. It was previously, however, not established whether the observed aggregation of cytochrome b5 occurred as an artefact of the isolation procedures or whether it was a specific interaction dependent on certain factors. In this chapter a number of bio-physical analytical tools was utilised to further elucidate the phenomenon of cytochrome b5 aggregation *in vitro*.

Although ESI-MS analyses previously confirmed that the purified cytochrome b5 fraction contained only a single protein, SDS-PAGE analyses revealed multiple protein bands. Using different denaturing conditions we showed that these multiple bands could not be fully denatured to the monomeric form and the tetrameric form is particularly SDS resistant. As seen in figure 4.1, these different denaturing conditions yielded the same SDS-PAGE protein

profile. This finding suggested that the multimeric forms of cytochrome b5 are very stable complexes, as a control protein (human phosphopantothenoyl cysteine decarboxylase) fully dissociated to its monomeric state using the conventional denaturing conditions and Laemmli® sample buffer.

During Western blot analyses, antibodies raised against cytochrome b5 predominantly recognised both the monomeric and tetrameric form of the protein. Isolating the antibodies for these forms and reusing it for Western blot analyses showed that the immunoglobulins isolated against the tetramer specifically only recognised that form whilst the immunoglobulins recognising monomeric cytochrome b5 cross-reacted with the tetrameric cytochrome b5 (figure 4.2). This result indicated that the conformation of the cytochrome b5 changed significantly upon aggregation and that the aggregated form was immunologically distinct from the monomeric form.

Furthermore, after separation of cytochrome b5 using SDS-PAGE, the monomer and tetramer was excised from the stained gel. These excised bands were digested by trypsin and the resulting peptide fragments identified using MS/MS. The results revealed that both excised bands contained peptides with sequences corresponding to that of cytochrome b5 (table 4.1).

A fluorescent dye ANS was used to investigate the concentration and ionic strength dependence aggregate formation of cytochrome b5. Heme containing proteins contain a hydrophobic pocket in which the heme is embedded, thus as a control a second heme containing protein (cytochrome C) was used. Cytochrome C showed no aggregation tendency as ANS fluorescence did not significantly increase with increase in protein concentration (figure 4.5), while an increase in cytochrome b5 concentration led to an increase in ANS fluorescence (figure 4.4). The oligomeric complex formation of cytochrome b5 was thus confirmed being both protein concentration and ionic strength dependent (figures 4.6 and 4.7).

Previous studies on cytochrome b5 showed that the membrane anchoring domain plays a crucial role in the aggregation of cytochrome b5[3][6]. By tryptic removal of this domain, the aggregation state of cytochrome b5 was abolished as indicated by the absence of fluorescence by the truncated form of the protein (figure 4.8).

The results presented in this chapter confirm earlier observations that cytochrome b5 forms stable oligomeric aggregates. The stability and specific dominance of the tetrameric

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complexes strongly indicated that a specific region or regions in the cytochrome b5 molecule were responsible for the formation of the tetrameric form of the protein observed *in vitro*. In Chapter 5 experiments are described to investigate a peptide sequence, identified by our group, as being responsible for aggregation. For this study, synthetic peptides were used to study secondary structure as well as interaction between the peptides and purified cytochrome b5. An enzyme linked affinity assay was developed to elucidate the interaction between purified cytochrome b5 and a synthetic peptide to gain insight into the aggregate formation of cytochrome b5.

Chapter 5

An investigation of the mechanism of cytochrome b5 aggregation

5.1   Introduction

During a study on the metabolism of progesterone in sheep adrenal microsomes, Swart *et al.* [78] also investigated the influence of cytochrome b5 on progesterone metabolism. Purified ovine cytochrome b5 and anti-cytochrome b5 serum were used to alter the cytochrome b5 levels in the ovine adrenal microsomes [78]. In these studies, cytochrome b5 addition had an apparent inhibitory effect on P450c17, while P450c21-activity appeared to be unaffected by changes in cytochrome b5 levels. No significant lyase activity towards progesterone could be detected in any of the experiments. These results were in contrast with results previously reported for guinea pig microsomes, where both P450c17 and P450c21 were stimulated by the addition of cytochrome b5 [79]. The investigators hypothesized that the selective influence of cytochrome b5 on sheep adrenal P450c17, shown in the study by Swart *et al.* [79], could explain the increase in cortisol to corticosterone ratios which occurred in certain animals upon hormonal stimulation. They concluded that this increase could only be due to an increased P450c17-activity, as the P450c21-activity was unaltered by changes in the cytochrome b5 levels in ovine adrenal microsomes. This observation also ruled out the possibility that cytochrome b5 interferes with the action of cytochrome P450 reductase.

The properties of ovine cytochrome b5 were subsequently investigated in our group, using SDS-PAGE, electrospray mass spectrometry and UV-VIS-spectroscopy to ascertain if the ovine derived enzyme differed significantly from the cytochrome b5 already characterised from other species [5]. The spectra of the purified ovine cytochrome b5 in the Soret region were identical to the spectra previously obtained for cytochrome b5 from other species. On SDS-PAGE the full-length protein exhibited a major protein band corresponding to a molecular mass of ±16 kDa, which represented the monomeric form of cytochrome b5 in accordance with previously published data [12]. Several bands corresponding to multimeric forms of the protein were, however, also observed. ESI-MS analyses of the same cytochrome b5 preparation, previously analysed by SDS-PAGE, indicated the presence of a single protein with a molecular mass of 15260 Da, corresponding to the molecular mass of *apo*-cytochrome b5. These findings indicated that the SDS-PAGE conditions, normally used for the resolution

of protein complexes, did not fully dissociate the cytochrome b5 aggregates. A truncated cytochrome b5, prepared by tryptic digestion, showed no aggregation and yielded only a single band on SDS-PAGE corresponding to a molecular mass of ±10 kDa. The aggregation of cytochrome b5 was further confirmed by high performance gel permeation chromatography (HP-GPC). HP-GPC analyses of the purified protein, under conditions of low ionic strength (10 mM TRIS, 0.1 mM EDTA, pH 8.15), yielded two peaks with both fractions exhibiting a typical cytochrome b5 absorption spectrum. These two fractions were collected and immediately rechromatographed under the same conditions. Each sample produced a single peak. When the same two fractions were incubated for 24 hours before they were rechromatographed, the higher molecular mass component dissociated into two fractions while the lower molecular mass fraction remained a single peak. Under conditions of higher ionic strength, only the higher molecular mass aggregated form of cytochrome b5 was observed in HP-GPC analyses [3]. The induction of higher molecular mass aggregates of cytochrome b5 by high salt or protein concentrations agreed with data previously published by Calabro and Katz [80]. Western blot analyses of adrenal and testicular tissue from different species showed that the cytochrome b5 was present in an aggregated 64 kDa form with little or no monomer being detected. From these studies we concluded that, apart from the aggregation to form specific 64 kDa tetramers, the ovine cytochrome b5 exhibited no anomalous structural characteristics.

As discussed earlier, recent studies in our group revealed that the linker and the membrane anchoring domains play a pivotal role in the oligomerisation of cytochrome b5. Using fluorescence resonance energy transfer (FRET) studies Storbeck *et al.* [6] showed that cytochrome b5 formed homomeric complexes in COS-1 cells and that the linker domain plays a vital role in the formation of these complexes. In our studies, it was shown that in COS-1 cells expressing full-length cytochrome b5 complex formation could be observed using FRET. Also, when co-expressing full-length cytochrome b5 with P450c17, it was observed that the protein predictably augmented the lyase activity of P450c17. With the truncated form of the protein, FRET studies revealed that minimal fluorescence could be observed [6] confirming that removal of the membrane anchoring domain decreased aggregation of the protein considerably [3]. Furthermore, co-expressing the truncated form of cytochrome b5 with P450c17 also had a stimulatory effect on the lyase activity of the P450 enzyme, although less than 50% conversion of the substrate was observed when compared to incubation with full length cytochrome b5 [6]. It was also shown that, without the globular N-

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terminus head domain, almost the same conversion of 17-OH-PREG to DHEA could be obtained as for the truncated form. For a form containing only the membrane anchoring region and the linker domain, the same observation was made. FRET also showed that the construct containing only the membrane anchoring domain and the linker also forms complexes *in vivo* [6].

In this chapter a synthetic peptide, corresponding to the hinge domain, was used to investigate its association with purified cytochrome b5. The synthetic peptides which were used were the following: a wild-type peptide (IITTIDSNSS) and a control peptide; (LLSSLKAVAV). Circular dichroism was used to determine whether this synthetic peptide, and its control, had any regular or irregular structure in solution. Also, a modified enzyme linked immunosorbant assay (mELISA) was developed for studying the association of the linker with the protein. The mELISA was used to establish if peptides were able to associate with purified protein, whether association was specific and if saturation could be obtained between the protein and a biotinylated-peptide. The biotinylated-peptides contained the same sequences but were conjugated, at the N-terminus, to biotin via an aminohexanoic acid linker.

5.2   Circular Dichroism Spectroscopy

Circular dichroism (CD) measures the difference between the absorption of left– and right circular polarised light which arise due to structural asymmetry of molecules. Circular polarised light are interconvertible with plane-polarised light. The latter beam consists of right– and left circularly polarised beams of equal intensities, where as the former has two orthogonal plane-polarised beams which are 90 degrees out of phase. The absorbance for the left-circular polarised light can be given by the equation;

$$A_l = \log_{10} (I^0_l/I_l) = \varepsilon_l C l$$

where I^0_l and I_l are the intensities of left-circular polarised incident on the sample and travelling distance l through a medium which contains a molar concentration (C) of a chiral solute. A corresponding equation can be used for the right-circular polarised light [81].

Modern instruments, which measure CD, using a modulation technique, generally measure a change in absorbance which is very small. Thus, for logical reasons many use $\Delta\varepsilon$ to report CD data. However, this is not the only measure of CD and numbers of alternatives are used especially in the biochemical literature. The first method of measuring CD took advantage of the fact that, when passed through a circular dichroic medium, the differential absorption of

the two circular components converts the plane-polarised light into elliptically polarised light. When the electric vectors of these two components are in the same direction, the sum of their magnitudes yields the semi-major axes of the ellipse, where as when they are in the opposite direction, the difference yields the semi-minor axes. Ellipticity (θ) can be characterised using these axis by taking the ratio of the semiminor and semimajor. This ellipticity is directly proportional to CD and is widely used in literature. Moreover, CD spectra in biochemical literature are often reports the data in terms of molar ellipticity ([θ]). This conversion, from ellipticity to molar ellipticity, is relatively simple. Despite the scale for presenting CD data it is also common practise to use different forms of ellipticities for different regions of the wavelength spectrum. When working in the near-UV region one often reports the data in terms of protein molarity while when working in the far-UV region the data is reported in terms of mean residue concentration; the latter is commonly used for polypeptides and proteins. The mean residue is calculated as the product of the molar concentration and the number of amino acids of the protein.

5.3   Experimental Procedures

5.3.1 Circular Dichroism analysis of peptides

5.3.1.1 Accurate weighing of wild-type –and control peptides

Ca. 1.5 mg peptide was measured in a clean, pyrolised glass vial and dissolved in ultra pure water (500 µL). The suspension was lyophilised overnight where after it was placed in a desiccator, containing a vial of phospho-pentoxide as a desiccant. The peptides were weighed within an hour after lyophilisation on a 6 decimal scale (Metler Toledo), containing a vial of phospo-pentoxide placed 60 minutes prior to weighing. The pre-weighed vials containing the peptides' weight were recorded every minute, for 12 minutes, and the last three recordings were averaged for the accurate mass of the peptides. The peptides were stored at ambient temperature in a desiccator containing phospho-pentoxide before CD analysis.

5.3.1.2 The effect of concentration on the structure of the peptides

As discussed in Chapter 4, protein concentration has a major effect on the aggregation of cytochrome b5. We investigated the concentration effect using the wild-type decapeptide (IITTIDSNSS), corresponding to the membrane anchoring domain, and a modified control decapeptide (LLSSLKAVAV) (GL Biochem LTD, Shanghai). Peptides were dissolved in 1

mL 20% acetonitrile in water. This stock solution was diluted to 200.0 µg/mL and 600.0 µg/mL solutions for both the wild-type and the control peptides. These solutions, 100 µL, were carefully transferred to a 0.5 mm quartz cuvette and subjected to CD analyses on Applied Photosystems Chirascan Plus spectropolarimeter. The instrument settings were as follows: scan range from 260 – 185 nm; bandwidth, 0.5 nm increment; scanning time per point, 0.5 seconds/point; temperature, 20°C; with three repeats per analyses.

5.3.1.3 The effect of ionic strength on the structure of the peptides

It was previously shown that the ionic strength plays a pivotal role in the complex formation of cytochrome b5. The effect of ionic strength on the structure of the two peptides, was investigated by making dilutions with diluents of increasing ionic strength. Using a sodium phosphate buffer (pH 7), the peptide stock solutions were diluted to 200 µg/mL with solutions containing 10, 30, 60 and 80 mM phosphate buffer respectively (see table 1). These solutions were incubated for 15 minutes at ambient temperature followed by CD analysis under the same conditions as described earlier.

Table 1: Preparation of the different salt concentrations used to study the effect of ionic strength on peptide structure. The buffer stock concentration was 100 mM.

[Phosphate buffer]	10 mM	30 mM	60 mM	80 mM
Buffer (µL)	10	30	60	80
20% ACN (µL)	70	50	20	0
Wild-type peptide Stock (µL)	20	20	20	20
Buffer (µL)	16	48	96	128
20% ACN (µL)	124	92	44	12
Control peptide Stock (µL)	20	20	20	20

5.3.2   Association of the peptides with cytochrome b5

To investigate whether the synthesised peptide associated with the purified protein, an *in vitro* study with the components was developed. A modified enzyme-linked immunosorbant assay (mELISA) was developed for this study.

The mELISA developed for the purpose of this study is shown in figure 5.1. The protein, cytochrome b5, was immobilised on an ELISA Nunc® 96-well microtiter plate, followed by the addition of a biotinylated control or wild-type peptide (peptide from GL Biochem LTD, Shanghai). If the hinge region is involved in cytochrome b5 aggregation, the biotinylated

peptide will associate with the hinge region of the immobilised hemoprotein. An avidin labelled horseradish peroxidase (HRP) is added to associate with the biotin labelled peptide and the amount of peptide immobilised can be quantified by the increase in absorbance at 405 nm after addition of substrate for HRP.
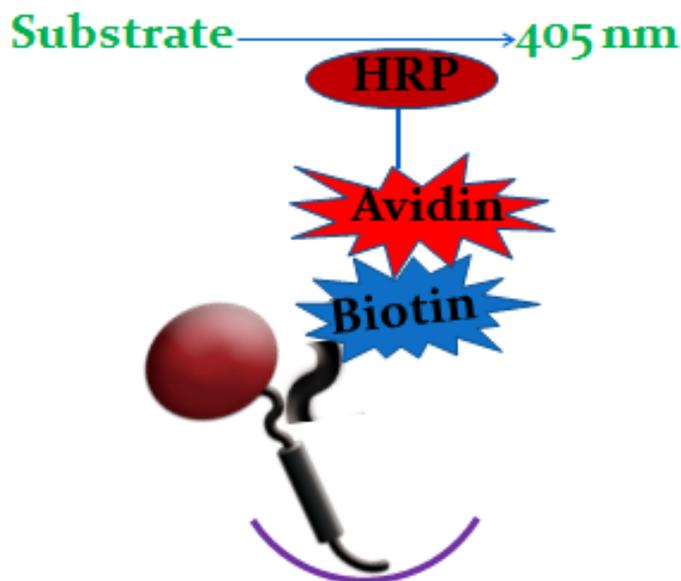
Figure 5.1: Schematic representation of the mELISA for cytochrome b5-peptide interaction. The assay involves the immobilisation of cytochrome b5, followed by the incubation with a biotinylated-peptide where after the HRP-avidin conjugate is applied. An appropriate substrate is added for colorimetric visualisation (Biotin and streptavidin has one of the strongest non-covalent interactions known in nature. The complex has a calculated dissociation constant (K_d) of 10^{-14} mol/L [82].

5.3.2.3 Specificity of biotinylated peptides for cytochrome b5

Before any experiments could be completed with the wild-type peptide, a number of control experiments had to be completed. These controls included an experiment where the specificity of the peptide for our protein of interest, cytochrome b5, was investigated. As a test for specificity, 25 – 1000 ng of cytochrome b5, lysozyme and BSA were respectively immobilised on a NUNC^{TM} 96-well microtiter plate by incubating 200 µL of 50 mM carbonate buffer, pH 9.0 containing the appropriate amount of each protein, in each well for 16 hours at 4° C. After coating the plate with the protein, the solutions were decanted from each well and all unoccupied spaces were blocked by adding 200 µL casein buffer (10 mM TRIS-HCl, pH 7.6, 0.15 M NaCl, 0.5% casein and 0.02% Thiomersal). After 60 minutes at

37°C, the content of each well was decanted and the wells were washed using 200 µL PBS-Tween-20 (PBS containing 0.1% Tween -20) followed by the addition of 100 µL biotinylated peptide (GL Biochem LTD, Shanghai) in PBS (2.7 µg peptide) to each well. The plate was incubated for 90 minutes at 37°C, where after all solutions were decanted. The plate was subsequently washed 3 times with 200 µL PBS-Tween-20, followed by the addition of 100 µL Avidin-HRP conjugate (1: 5000 in PBS) to all the wells. The plate was incubated for another 60 minutes, solutions was decanted and the plate was washed as described earlier. Substrate solution (100 µL of 6 mg 2-2'-azino-di-(3-ethylbenzthiazoline sulfonic acid), 6 µL 32 % H_2O_2 in 12 mL citrate buffer, pH 5.0) (ABTS) was added to each well, the solution was allowed to react for 15 minutes at 37°C and the absorbance subsequently measured at 405 nm.

In addition a constant amount of cytochrome b5 (500 ng) was immobilised as described above and the mELISA repeated using the wild-type biotinylated peptide as well as the control biotinylated peptide.

5.3.2.4 Saturation curve using both wild-type -and control biotinylated peptides

After establishing that the biotinylated-peptide associates specifically with cytochrome b5, saturation studies were performed using the mELISA. Cytochrome b5 and BSA, 1 – 2000 µg, were respectively immobilised on a NUNC™ 96-well microtiter plate. For BSA a stock solution of 10 mg/mL was used and for the cytochrome b5 a 1 mg/mL stock solution. Where necessary, cytochrome b5 was concentrated to *ca.* 200 µL using a SpeedyVac Sample Concentrator. For 600, 1000 and 2000 µg protein, 600, 1000 and 2000 µL of the stock solution were used to concentrate. A detailed description of the protein immobilisation is given in Table 2.

Table 2: Immobilisation protocol for BSA and Cytochrome b5 on a NUNC™ 96-well microtiter plate for the mELISA.

µg Protein Immobilised	1	10	75	150	300	600	1000	2000
BSA (10 µg/µL Stock) (µL)	0.1	1	7.5	15	30	60	100	200
Buffer (µL)	200	199	192.5	185	170	140	100	0
Cytochrome b5 (1 µg/µL Stock) (µL)	1	10	75	150	300	200	200	200
Buffer (µL)	199	190	125	50	0	0	0	0

*Shaded samples: Samples which were concentrated using SpeedyVac Sample Concentrator.

After incubating the plate for 16 hours at 4°C the solutions were decanted and the wells treated for 60 minutes, with 200 µL casein buffer to prevent non-specific binding. The casein was discarded and the wells were washed once using PBS-Tween-20 followed by the addition of the biotinylated peptides in PBS (2.7 µg), as described earlier. Incubation steps and substrate addition were performed as described in section 5.3.2.3.

5.4 Results

Recently our group investigated complex formation of cytochrome b5, using FRET. It was found that the membrane anchoring domain played a pivotal role in the aggregate formation of the protein. From these findings, through different mutations in the membrane anchoring domain, it was deduced to use the portion of the membrane anchoring domain identified as IITTIDSNSS. From the sequence of the wild-type peptide containing branched amino acids on the N-terminus side would be more prone to beta-sheet formation. As for most beta-sheet peptides, this peptide was also found to be quite insoluble in water, which resulted in the use of 20% acetonitrile to solubilise the peptide. The control peptide (LLSSLKAVAV), however, was designed with conservative mutations on the N-terminus to be more prone to form alpha-helices and random structures. Also, the C-terminus of the peptide was mutated to limit hydrogen bonding of side chains, as well as electrostatic interactions with cationic groups.

5.4.1 Circular dichroism of Peptides

The ellipticity values obtained for each sample in the CD spectrum are in millidegrees ellipticity (θ). These values can be standardised and expressed, either as molar ellipticity ([θ]) or as the mean residue ellipticity. The former can be calculated using the molar concentration of each solution, its ellipticity at each wavelength (λ) and the path length of the cuvette. The equation for this conversion is given by:

$$[\theta]_{\text{molar}, \lambda} = \frac{\theta \times 100}{[M] \times l}$$

where [M] is the molar concentration of the peptide and l is the path length of the cuvette in centimetres. Molar ellipticity, [θ], is given in *degrees cm^2 dmol^{-1}*.

The secondary structure of protein and peptides can be studied using CD [83]. Secondary structures such as α-helices, β-sheets, etc. yield different CD spectra as can be seen in figure 5.2. Even random/irregular structures can be detected using this method.
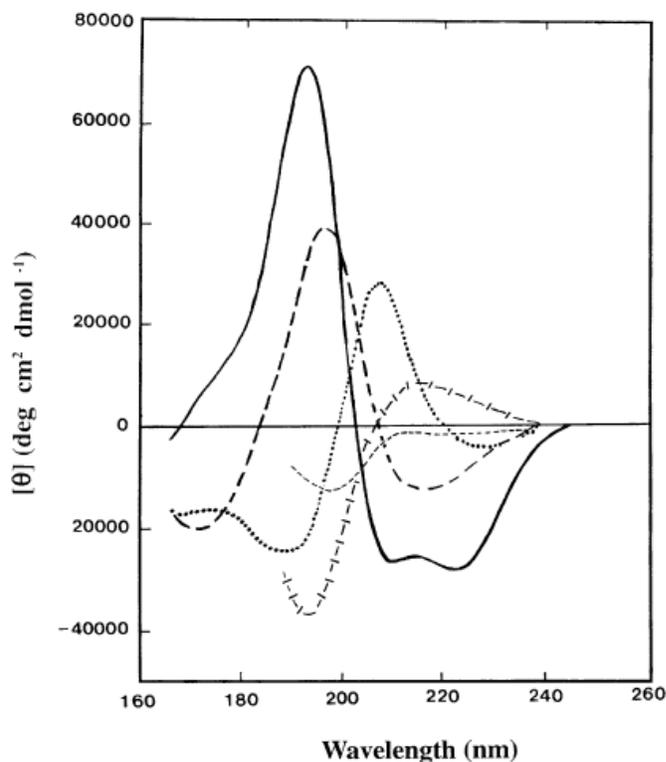
Figure 5.2: Circular dichroism spectra for various protein secondary structures. Spectra represent: solid line, α-helix; long dashed line, anti-parallel β-sheet; dotted line, type 1 β-turn; crossed dashed line, extended 3_{10}-helix or poly (Pro) II helix and short dashed line, irregular structure [83].

5.4.2 Effect of concentration on the structure of peptides

Figures 5.3 and 5.4 shows the CD spectra for the wild-type and control peptides at low peptide concentrations (200 µg/mL) and at relatively high peptide concentrations (600 µg/mL). The CD spectra (figure 5.3) indicate that both peptides at 200 µg/mL are represented by irregular structures in PBS. Such irregular structures are characterised by an intense ellipticity minimum at ~195 nm (refer to figure 5.2). For the wild-type peptide two different minima could be observed. As for the control peptide, the minimum at 195 nm indicated the presence of irregular structures, however, the intensity was 50% less than that of the wild peptide. This indicated the wild-type peptide had less irregular structures. The shoulder minimum at ~220 nm, indicated a secondary structure such as a β-sheet or an α-helix. However, as this wild-type peptide contains Thr and Ile which both have β-branched side chains that do not permit α-helices, it is more plausible that the ordered structures may be β-sheets. The fact that the wild-type peptide was quite difficult to get into solution and tended to form aggregates also pointed to a tendency to form β-sheet structures which tend to be less soluble.
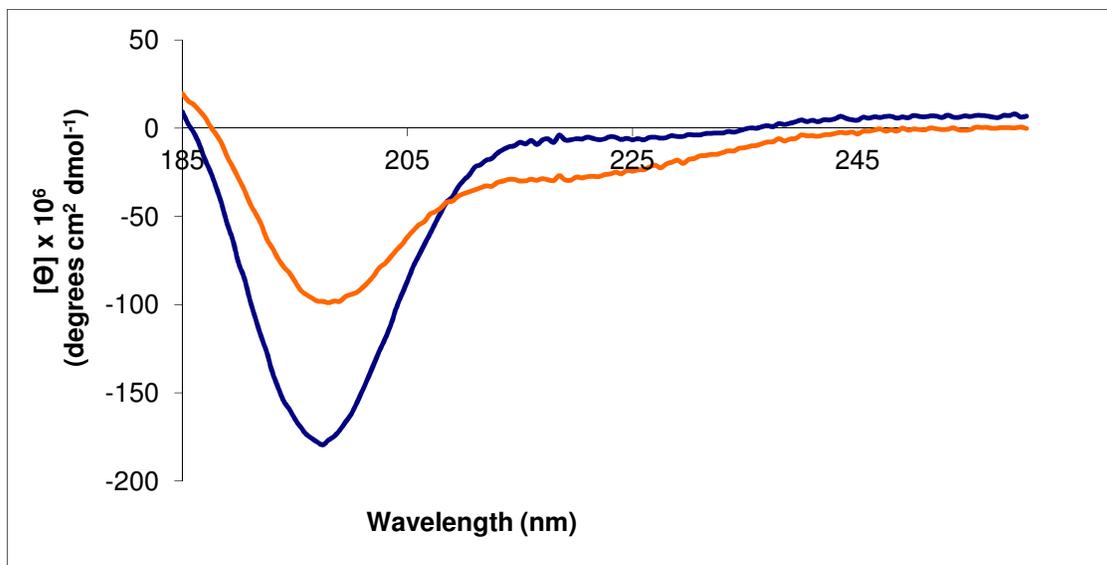
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Figure 5.3: Far UV CD spectra for the wild-type and control peptides (200 µg/mL). Orange and blue solid line represents CD data for wild-type and control peptides, respectively.
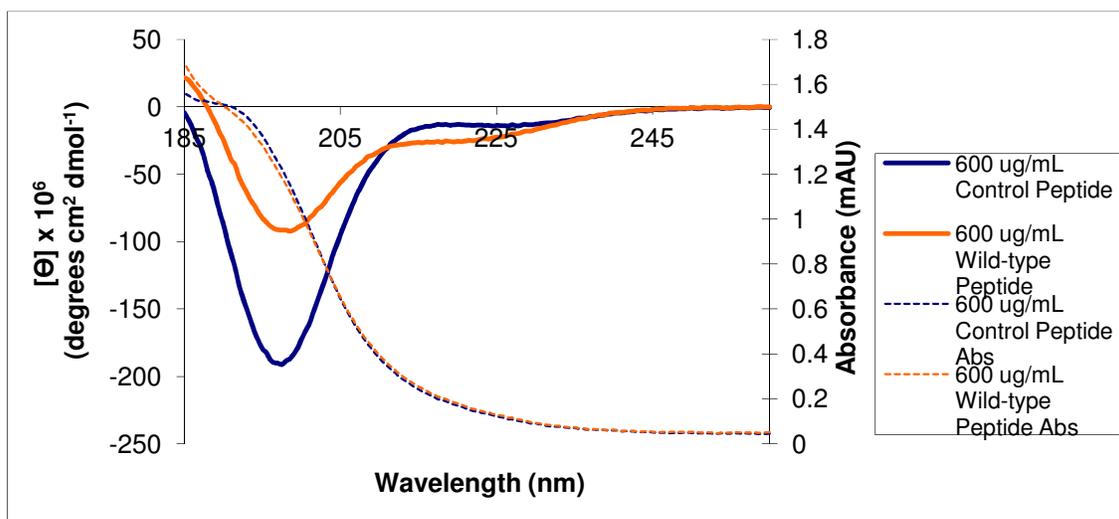
Figure 5.4: Far UV CD spectra for the wild-type and control peptides (600 µg/mL). Also shown on secondary y-axis is the absorbance of each peptide. Orange and blue solid line represents CD data for wild-type and control peptides respectively, while dashed line represents their absorbance.

5.4.3 The effect of ionic strength on the structure of the peptides

By dissolving the peptides in solutions containing increasing concentrations of phosphate buffer, it was observed that the difference in ionic strength had a slight effect on the CD spectrum of peptides. This was in contrast to what was found in the previous chapter using the ANS dye and purified cytochrome b5. It was expected that the wild-type peptide would

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show signs of more organised structure as the ionic strength was increased. For both the peptides there was a slight loss in the 195 nm ellipticity at about 10% for the wild-type peptide and >20% for the control peptide (Figures 5.5 and 5.6). As the wild-type peptide was already more ordered than the control peptide the change in ionic strength probably had less of an affect. The control peptide adapted more ordered structures in the higher ionic strength buffers, as such an ionic environment leads to dehydration.
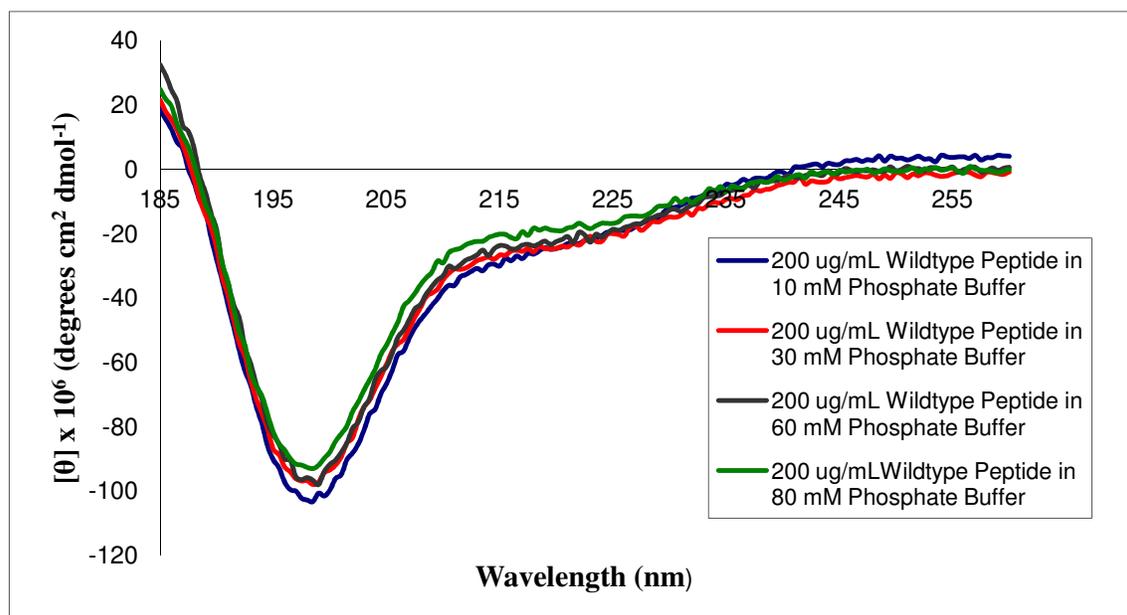
Figure 5.5: Far UV CD spectra for the wild-type peptide (200 ug/mL). The peptide was titrated with solutions of increasing ionic strength (10 – 80 mM phosphate buffer).
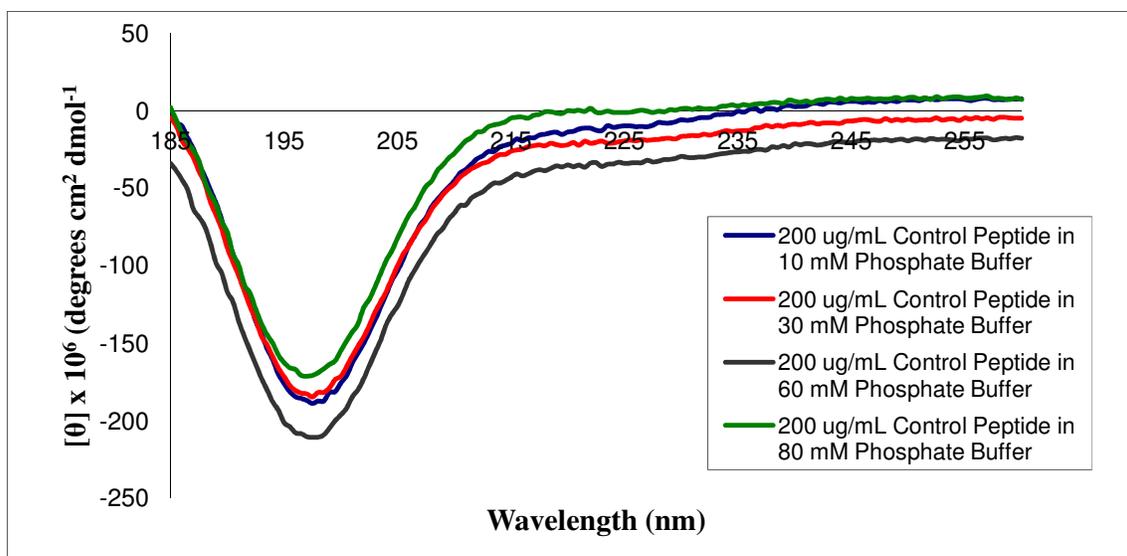
Figure 5.6: Far UV CD spectra for the control peptide (200 ug/mL). The peptide was titrated with solutions of increasing ionic strength (10 – 80 mM phosphate buffer).

5.4.4 Association of biotinylated-peptides with cytochrome b5

Interaction of both the wild-type and the control peptides with 500 ng of immobilised cytochrome b5 was tested using the mELISA and biotinylated versions of the two peptides. (figure 5.7). It is clear that the control peptide has a significantly lower attraction (P=0.012) for the cytochrome b5 than the wild-type peptide.
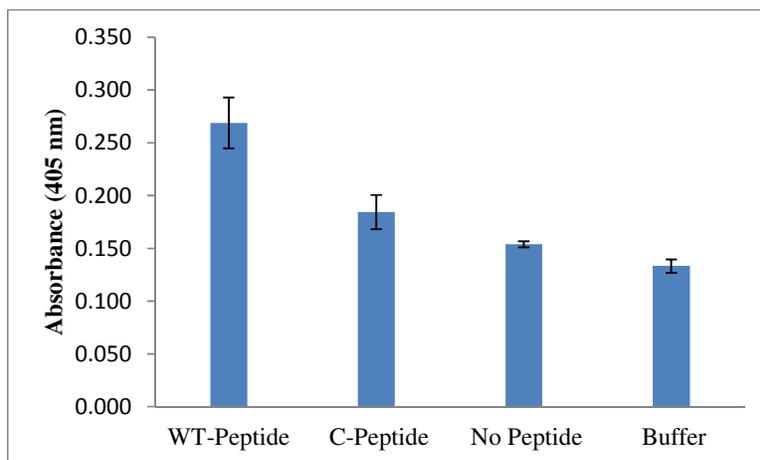
Figure 5.7: Affinity of biotinylated-peptides for immobilised cytochrome b5. Biotinylated-peptides (2.7 µg) were incubated with 500 ng immobilised cytochrome b5 where after streptavidin-HRP conjugate (1: 5000) was added. Wt-Peptide; wild-type peptide, C-Peptide; control peptide, No Peptide; No peptide added to 500 ng cytochrome b5 and Buffer; no cytochrome b5 immobilised.

The specificity of the wild-type peptide for cytochrome b5 was also determined by using cytochrome b5, lysozyme and BSA (Figure 5.8).

As expected, there is a non-specific concentration independent interaction between the BSA and lysozyme and the biotinylated peptide. This could be due to non-specific hydrophobic interactions with BSA and its hydrophobic pockets and the increase in absorbance for the lysozyme could be a result of this cationic protein interacting with the anionic peptides. However, as the amount of protein was increased, only the signal for the cytochrome b5 increased in a concentration-dependent manner indicating a higher affinity specific interaction between the wild-type peptide and cytochrome b5 (figure 5.8). The concentration depended increase of cytochrome b5 could indicate that much more specific electrostatic interactions, such as hydrogen bonding, could be in play, rather than hydrophobic and ionic interactions. These bonds are probably hidden and quite stable and not disrupted by the detergent wash steps, as for the non-specific binding which occurred between the peptide and the control proteins.
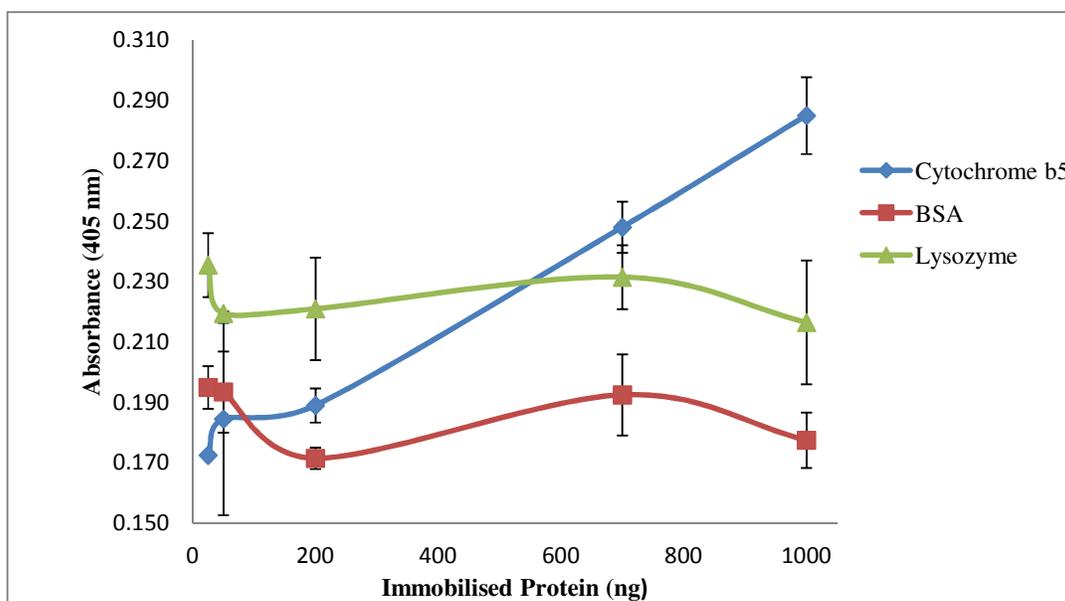
Figure 5.8: Specificity of biotinylated wild-type peptide. The biotinylated-peptide (2.7 µg) was incubated with increasing amount of different proteins (indicated in legend). The presence of the bound biotinylated-peptide was determined using streptavidin-HRP conjugate (1: 5000).

5.4.5 Saturation curve using both wild-type -and control biotinylated peptides

Establishing that the peptide associates with cytochrome b5, we investigated whether binding saturation could occur at 37°C by increasing the amount of antigen and keeping the ligand (biotinylated-peptide) concentration constant. As shown in figure 5.9, both the wild-type and the control peptides reached saturation. This indicated that the N-terminus sequence of the wild-type peptide IITTI and the conservatively mutated control peptide sequence of LLSSL, may both be able to interact with cytochrome b5, possibly via hydrogen-bonding with the hydroxyl groups in the side chains. The C-terminus sequence of the wild-type and control peptides are very different namely DSNSS and KVAVAV, respectively. The C-terminus of the wild-type peptide can form multiple hydrogen bonds, while that of the control peptide can form non-specific hydrophobic interactions. Also, the cationic control peptide may have electrostatic interaction with the Asp in the hinge region. It was, however, clear that the wild-type peptide reached higher saturation values at a lower immobilised protein amount with 50% saturation reached at 399 µg immobilised cytochrome b5 *versus* 540 µg for the control peptide. No increase in baseline association was observed for the BSA control protein with either control or the wild-type peptide.
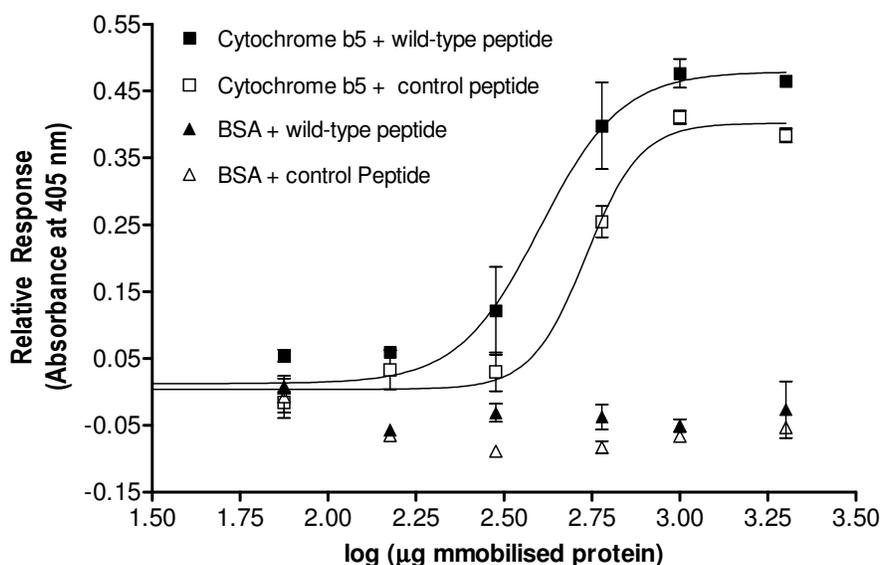
Figure 5.9: Saturation binding curves of biotinylated-peptides. Biotinylated-peptides (2.7 µg) were added to increasing amounts of immobilised protein. The presence of the biotinylated-peptide was determined using streptavidin-HRP conjugate (1: 5000) in duplicate analyses.

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5.5 Conclusion

Our group has shown that the full length form of cytochrome b5 is able to form multimeric complexes [3] when purified as described in literature [4]. Lombard *et al.* [3] showed that these multimeric complexes are dependent on the membrane anchoring domain. The investigators showed, by purifying cytochrome b5 from ovine liver and analysing the purified fraction using both SDS-PAGE and high performance gel permeation chromatography (HP-GPC), multimeric complexes could be observed. Furthermore it was shown, when removing the membrane anchoring domain through tryptic digestion, that the appearance of these multimeric complexes was abolished [3].

In general small non-constrained peptides show very little organised structure in normal buffers. We also observed, using CD, that the two decapeptides in the study had predominantly unordered structures. However, when the two peptides were directly compared, the wild-type peptide had more ordered structure, possibly β-sheets, with less of an irregular structure than that of the control peptide. Titration of the peptides using sodium phosphate salt had very little effect on the structure of the wild-type peptide (figure 5.5). Conversely, the control peptide showed that as the salt concentration increased, less irregular structure was observed (figure 5.6). In the fluorescent studies (Chapter 4), we observed that the ionic strength had a definite effect on the aggregation. This was, as the ionic strength increased so did the aggregation (section 4.2.4). For those studies it was necessary to increase the salt concentrations to more than 500 mM. Unfortunately, such high concentrations cannot be reached using CD as this would result in interference in the far UV region.

We investigated whether or not the synthetic peptides were able to associate with purified cytochrome b5 *in vitro*. The wild-type peptide interacted significantly (P=0.012) better with cytochrome b5 than the control peptide (figure 5.7). This interaction was also highly specific for cytochrome b5, with no specific affinity for BSA or lysozyme (figure 5.8). From figure 5.9 it can be seen that both the wild-type and the control peptide had saturating binding to immobilised cytochrome b5, but the control peptide had a lower affinity and bound to a lesser extent. These results revealed that the wild-type peptide had a specific affinity for the purified cytochrome b5, which probably depended on a hydrogen bond network.

Chapter 6

Conclusion

Cytochrome b5 can be found in various mammalian tissues as a soluble cytosolic protein, consisting of 98 amino acids expressed primarily in erythrocytes, as well as an amphipathic membrane bound protein. The amphipathic membrane bound cytochrome b5 is divided into two types, depending on primary structure and sub-cellular localization. The first, a 134 amino acids form of cytochrome b5, is found in the endoplasmic reticulum and is the only form known to significantly affect adrenal steroidogenesis [1], [84]. The second form of cytochrome b5 is localized primarily to the outer mitochondrial membrane and consists of 146 amino [84–88].

The influence of microsomal cytochrome b5 on adrenal steroidogenesis, and more specifically the 17,20-lyase reaction catalyzed by cytochrome P450 17α-hydroxylase/17,20-lyase, has become a topic of interest due to the clinical importance of this key steroidogenic monooxygenase [89]. Results from our laboratory have also recently shown that cytochrome b5 augments the activity of 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase, further expanding the role of this hemoprotein during steroidogenesis [44], [46].

Previous studies in our laboratory on the enzymatic properties of ovine cytochrome b5, revealed that the enzyme exists in more than one aggregation state *in vivo* [90]. The aim of this investigation was to isolate and purify ovine cytochrome b5 for subsequent identification and characterization of the regions in the molecule responsible for the previously observed aggregation.

Cytochrome b5 was previously isolated by Lombard *et al.* [3] using a PEG fractionation method. In this study the same method was employed for the isolation of cytochrome b5 from ovine liver. Purification data revealed (Table 3.2) that a 118-fold purification could be achieved with a yield of 55%. During the course of this study, the purification method for cytochrome b5 had to be scaled up to increase the yield of the protein in order to obtain data with physical analytical techniques to further elucidate aggregation. The data gathered throughout this purification showed that similar yields could be achieved but that the fold purification decreased by more than 50% (Table 3.3). For this study, vast amount of cytochrome b5 was needed and the up-scaling of the technique was appropriate. In terms of

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specific activity, up-scaling is not recommended as there is no increase in the fold purification after the second purification step.

During analysis of a microsomal suspension, using SDS-PAGE, a prominent protein band could be observed at ±60 kDa and no apparent protein band could be observed corresponding to the reported molecular weight of cytochrome b5 (16 kDa). The SDS-PAGE protein profiles for all three the purified fractions, however, showed that cytochrome b5 was purified with protein bands corresponding to a molecular weight of 16 kDa (monomer). This protein band was accompanied by an additional band corresponding to ± 60 kDa (tetramer) in all purified fractions. Other multimeric complexes could also be observed, albeit to a lesser extent. These multimeric complexes were recognized by cytochrome b5 antibodies and were concluded to be dimers (32 kDa), trimers (48 kDa), etc. In terms of antibody specificity, both forms of the protein were recognized. To a larger extend the monomer and the tetramer (Figure 3.3).

The purified protein was subjected to ESI-MS for accurate mass determination. Although SDS-PAGE revealed multiple protein bands, MS analysis indicated only a single heme containing protein (Figure 3.6). The *apo*-protein detected had a molecular weight of 15249 Da (Figure 3.7), similar to that previously reported by Lombard *et al.* [3]. Due to the absence of the higher molecular weight proteins it was concluded that the ionization process in the mass spectrometer abolished the existence of all aggregates in solution, thus leaving only a monomeric form of the protein to be detected.

For the preparation of protein samples for separation using SDS-PAGE, the protein solution is treated with a treatment buffer containing both sodium dodecyl sulphate and 2-mercaptoethanol. The former is an anionic detergent, which disrupts non-covalent bonds present in protein resulting in the loss of protein conformation, while the latter is a reducing agent responsible for breaking disulphide bonds. Even with these two components present in the treatment buffer, the multiple bands for purified cytochrome b5 on the polyacrylamide gel persisted. The same results were obtained through the addition of a third denaturing agent, 8M urea, together with denaturing conditions commonly used for preparing membrane bound proteins for SDS-PAGE analyses. During the investigation of different denaturing conditions, human phosphopantothenoyl cysteine decarboxylase was included as control. This protein exists as a homomeric trimer with a molecular weight of 75 kDa [91]. Using conventional denaturing conditions it was possible to fully denature this trimer protein into its monomeric

form and this could be observed at a molecular weight of 25 kDa on the polyacrylamide gel (Figure 4.1). These results further confirmed that cytochrome b5 formed specific highly stable tetrameric aggregates.

Immunological studies revealed that the antibodies, raised specifically against the aggregate from of cytochrome b5, showed affinity for only the aggregated state while the antibodies raised against the monomeric form recognised both the monomer and the aggregate (Figure 4.2). This was in contrast to what was found by Storbeck [6] who showed that the antibodies raised against the aggregate recognised both forms of the protein while the monomeric antibody only recognised the monomer. There can be two possible explanations for this finding: (a) because both the monomeric and aggregate antibodies were isolated from the same immune serum, the chances for cross recognition were high and almost inevitable and (b) because titres were not determined, isolation of these specific antibodies with the use of only three steps, as described by Lombard *et al.* [3], could also lead to cross contamination if three isolation steps are not sufficient.

A further investigation of the proteins in the two bands (corresponding to 16 and 60 kDa respectively), observed during SDS-PAGE analyses of cytochrome b5, included the excision of the protein bands from the SDS-PAGE gel and identification of the proteins using MS/MS. The excised bands were digested with trypsin and the resulting peptides were identified using a database search (Matrix Science - Mascot MS/MS Ions Search). Both the bands, corresponding to the molecular weights of 16 and 60 kDa, were identified to have peptide sequences indicative of cytochrome b5. These bands were identified with a high degree of certainty as the results obtained had Mascot Scores well above 20 (a score of 20 and above considered to be significant) (Table 4.1) [92]. These results confirmed that the denaturing process commonly used for SDS-PAGE analyses did not fully dissociate the aggregates formed by cytochrome b5 and confirmed earlier observations by Lombard *et al.* [3].

Previously the effect of ionic strength on aggregation was investigated [3]. Using high performance gel permeation chromatography (HP-GPC), Lombard *et al.* [3] showed that two peaks could be observed when analysing purified cytochrome b5. Both these peaks exhibited typical cytochrome b5 spectral properties. Changing the mobile phase to PBS, they found that only the single peak could be observed. This peak had the same retention time as that of the higher molecular weight (64 kDa) form of the protein.

In our study we investigated the effect of ionic strength on purified cytochrome b5 using fluorescence. A fluorescent dye, 1-anilinonaphthalene-8-sulfonic acid (ANS), was used which fluoresces when exposed to hydrophobic areas. Preliminary studies showed that a definite increase in the fluorescence could be observed for cytochrome b5 as the protein concentration was increased (Figure 4.4) while the fluorescence for cytochrome C remained at a basal level under similar conditions (Figure 4.5). This observation indicated that the aggregated form of the protein was present. In addition, by incubating 100 µg cytochrome b5 in different buffer solutions, with ionic strengths ranging from 5 mM – 1000 mM, an increase in the aggregation state of cytochrome b5 was observed as the ionic strength of the solution containing the protein was increased (Figure 4.7). This finding supported previous studies [3] which stated that the aggregation of the protein is dependent on the ionic strength.

The effect of protein concentration on the aggregation of cytochrome b5 was also investigated using the same fluorescent dye. Investigating the aggregation of cytochrome b5 using this dye has never been previously reported. An increase in the fluorescence could be shown with an increase in protein concentration, indicating a direct relationship between protein concentration and protein aggregation. No concentration dependent increase in fluorescence could be observed for the control cytochrome C under identical conditions (Figure 4.6).

During previous studies on cytochrome b5, Lombard *et al.* [3] showed that the hydrophobic membrane anchoring domain plays a crucial role on cytochrome b5's aggregation. This domain is susceptible to certain proteases and can be removed through trypsin digestion. Lombard *et al.* showed that, by removing this domain, the protein remained functionally active but, when analysed using SDS-PAGE, aggregation was completely abolished [3]. Using a truncated form of cytochrome b5, prepared as described by Lombard *et al.*, together with the ANS fluorescence dye, no fluorescence could be detected with an increase in protein concentration (Figure 4.8). This result agreed with the findings of Lombard *et al.* and confirmed the role of the membrane anchoring domain in aggregation [3].

Recently, Storbeck *et al.* [6] showed that cytochrome b5 forms aggregates *in vivo.* Using fluorescence resonance energy transfer (FRET), it was confirmed that the membrane anchoring domain is involved in the aggregation. By making certain mutations in the membrane anchoring domain, they were able to decrease the FRET signal significantly. The decrease in FRET provided sufficient evidence that this domain is involved in protein self

association. In this study, the membrane anchoring domain was investigated using a ten amino acid peptide and a control peptide. This decapeptide corresponded to a portion within the membrane binding domain. Circular dichroism was used to study the secondary structure of this peptide. We found that the wild-type peptide had 50% less irregular structure (Figures 5.3 and 5.4) than a control peptide (Figures 5.3 and 5.4). Also, structural analysis of the wild-type peptide included the appearance of secondary structures such as β-sheets and/or α-helices. Due to structural roles of Thr and Ile side chains, which contain β-branched side chains, it was concluded that the secondary structure observed could possibly be β-sheets rather than α-helices. None of these secondary structures were present in the control peptide. The effect of ionic strength on the secondary structure of the peptides was also investigated. Unfortunately, no significant difference could be observed for either peptide (Figures 5.5 and 5.6). This was in contrast with what was found in the fluorescence study. It is, however, important to note that in the fluorescent studies the ionic concentrations exceeded 500 mM. At such high ionic concentrations CD analyses could not be accomplished as a result of interference in the far-UV spectrum.

Association studies, using a modified ELISA technique, revealed that the peptide indeed associated with the purified protein in a specific manner. Binding studies revealed that saturation could be reached and that the wild-type peptide had a significantly lower dissociation constant than the control peptide (Figure 5.9). We found that the wild-type peptide had a significantly better interaction with cytochrome b5 than the control peptide. Furthermore, it was concluded that the interaction of the wild-type peptide is probably much more specific which is accomplished through hydrogen bonding. Binding studies also revealed that association of the control peptide with cytochrome b5 could be observed. This observation, however, showed that the wild-type peptide had higher saturation values and 50% saturation occurring at less than 25% immobilised cytochrome b5. Recently, using FRET, Storbeck *et al.* [6] also showed that the hydrophobic C-terminus plays a pivotal role in the protein's aggregate formation. The findings from this study concurred with Storbeck's conclusion, and the information gathered in the studies, using this peptide, strongly suggests that the hydrophobic membrane domain plays a specific role in the aggregation of cytochrome b5.

In this study we investigated the complex formation of cytochrome b5. The protein was isolated from ovine liver and partially characterised. The multiple bands present in SDS-

PAGE analyses showed us that the aggregated state of the protein was stable and could not be disrupted using conventional denaturing conditions. Furthermore, it was found that this aggregated state can be manipulated using either protein concentration and/or ionic strength. It was mentioned earlier that the membrane anchoring domain plays a role in the protein's aggregation and using a peptide corresponding to this region it was shown that this indeed was the case. This was accomplished through structural analyses and binding studies. This study also revealed that there was interaction between the peptide and the purified protein. This provided further evidence that the hydrophobic membrane anchoring domain plays a central role in the aggregation of cytochrome b5.

Cytochrome b5 is involved in a number of biological reactions. During these reactions it either provides the second, rate limiting electron to cytochrome P450 enzymes or cytochrome b5 enhances the activities of enzymes through an allosteric effect. Furthermore, there are several hypotheses postulated on how cytochrome b5 interacts with the enzymes which it augments. During the discussions of these hypotheses, it was concluded by a number of researchers that cytochrome b5 forms hetero-dimeric complexes with cytochrome P450 enzymes [1][90]. These complexes are described for both electron transfer reactions as well as allosteric effectors.

Our group has been isolating cytochrome b5 from animal tissue for a number of years and has observed and studied the aggregation for those years. In 2002, Lombard *et al.* raised the question "Sheep adrenal cytochrome b5: active as a monomer or a tetramer *in vivo*?" [3]. Apart from criticism that the aggregation was a result from the manner in which cytochrome b5 was isolated in our laboratory, it was also considered whether the protein could use this self association as a regulatory system *in vivo.* When Storbeck *et al.* [6] showed that cytochrome b5 aggregation could also be observed when expressed in living cells, it was confirmed that cytochrome b5 aggregation occurred *in vivo* and was not due to the isolation procedures followed in our laboratory.

The proposed mechanisms of action of cytochrome b5 are rather complex and discussed comprehensively in Chapter 2. During electron transfer reactions, it is considered that cytochrome b5 and cytochrome P450 form a two electron acceptor complex through charge pairing [1]. In addition, during allosteric stimulation, it is believed that cytochrome b5 and cytochrome P450 form a hetero-dimeric complex through charge pairing [1][90]. Investigating the aggregation of cytochrome b5 could therefore shed light on how

cytochrome b5 influences cytochrome P450-dependent reactions. The aggregation of cytochrome b5 can act as a regulatory mechanism. Self-association would inhibit augmentation of cytochrome P450-catalysed reactions as the formation of homomeric complexes prevents the cytochrome b5 forming heterodimeric complexes with cytochrome P450. Therefore, it can be considered that the monomeric form of cytochrome b5 would generally be considered the active form while the aggregated complex is the inactive form.

By considering that cytochrome b5 utilises this aggreagation as a method to switch between its active and inactive form, this could hugely impact on the steroid output in adrenal steroidogenises. This aggregation can thus be said to be a mechanism to control its association with P450 enzymes and 3βHSD which will, ultimately, influence the output of sex steroids, mineralocorticoids and glucocorticoids.

In this study we showed that self association can be manipulated using either protein concentration or ionic strength and that the membrane anchoring domain plays an important part in the formation of specific tetrameric complexes. Hence, it can be considered that the cell can employ these aggregation properties of cytochrome b5 as means of switching between the active and inactive forms.

Future studies on the aggregation of cytochrome b5 should investigate means to prove beyond doubt that aggregation is indeed used as a regulatory system for cytochrome b5 augmentation of cytochrome P450-catalysed reactions. These investigations could involve the permanent cross linking of cytochrome b5 monomers and investigating its function as an effector for cytochrome P450 reactions. In addition a physiological trigger for the aggregation and de-aggregation of cytochrome b5, coupled to a hormone signalling event, needs to be found in order to explain the differentiated effect of cytochrome b5 on cytochrome P450-dependent reactions in different tissues.

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