On the role of phospholipids in the cytochrome P450 enzyme system

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 9 september 1994
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

Het aardige van citaten is dat het lijkt of we enigzins bekend zijn met de auteur, wat sociaal vaak indrukwekkend is.

Kenneth Williams

Stellingen bij het proefschrift van W.G. Balvers.

I

De door Dutton *et al.* aangevoerde literatuur levert geen bewijs voor hun veronderstelling dat het semichinonimine-radicaal van resorufine wordt geprotoneerd op de stikstof en niet op de zuurstof.

Dutton, D.R., Reed, G.A. & Parkinson, A. (1989) *Arch.Biochem.Biophys.* 268: 605-616

II

Het bewijs van Bayerl *et al.* voor hun conclusie dat er een specifieke interactie bestaat tussen cytochroom P450 en fosfatidylethanolamine (PE) is onvoldoende en sluit niet uit dat er specifieke interacties bestaan tussen cytochroom P450 en fosfatidylserine (PS) en/of fosfatidylinositol (PI).

Bayerl, T., Klose, G., Ruckpaul, K. & Schwarze, W. (1985) *Biochim.Biophys.Acta* 812: 437-446

III

Het vergelijken van de resultaten van gepubliceerde studies over de rol van fosfolipiden in het cytochroom P450 enzym systeem is nagenoeg onmogelijk door de grote verscheidenheid aan gereconstitueerde systemen die zijn gebruikt.

Dit proefschrift (hoofdtuk 2 t/m 5)

IV

De manier van incorporatie van cytochroom P450 enzymen in gereconstitueerde modelsystemen is vergelijkbaar met de wijze van inbouw in het microsomale membraan

Dit proefschrift (hoofdstuk 2)

V

MO computerberekeningen zijn een zeer bruikbaar hulpmiddel bij het onderzoek naar de reactiemechanismen in de biochemie zolang de uitkomsten worden gebruikt op relatieve wijze.

VI

Door de onzekerheid in de correlatietijd (τ_c) en de spintoestand van de Fe^{3+} in de heem cofactor van cytochroom P450 zijn de met behulp van NMR-T_1 relaxatie metingen bepaalde afstanden tussen substraat atomen en het Fe^{3+}-atoom minder absoluut dan ze doorgaans worden gepresenteerd.

van de Straat, R., de Vries, J., de Boer, H.J.R., Vromans, R.M. & Vermeulen, N.P.E. (1987) *Xenobiotica* 17: 1-9

Castro-Maderal, L. & Sullivan , P.D. (1992) *FEBS Lett.* 296: 249-253

Myers, G.T., Thummel, K.E., Kalhorn, T.F. & Nelson, S.D. (1994) *J.Med.Chem.* 37: 860-867

VII

Het valt te betwijfelen of je eiwitten zoals cytochroom P450 en NADPH-cytochroom reductase, waarvan meer dan 85% van het eiwit zich in het cytoplasma bevindt nog wel integrale membraan eiwitten moet noemen.

Black, S.D. (1992) *FASEB J.* 6: 680-685

VIII

Wanneer de jacht op de walvis voor wetenschappelijke doeleinden doorgaat, zal dit dier de twijfelachtige eer te beurt vallen de eerste uitgestorven diersoort te zijn waarvan we alles weten.

IX

Bij de claim van de zuivelfabrikanten dat rechtsdraaiend melkzuur gezonder is dan linksdraaiend melkzuur wordt ook de waarheid 'verdraaid'.

Chemisch Magazine (april 1992)

X

Het 'voetje' dat wasmiddelenfabrikanten proberen te halen bij de consument is altijd witter dan wit.

Voorwoord

Dit proefschrift zoals het voor u ligt is het resultaat van vier jaar hard werken. Tijdens deze vier jaar heb ik echter ontzettend veel steun gehad van verschillende kanten die aanzienlijk hebben bijgedragen tot de tot stand koming van dit proefschrift. Deze mensen wil ik allemaal ontzettend bedanken.

Allereerst wil ik mijn promotor, professor C. Veeger bedanken omdat hij mij de gelegenheid heeft geboden dit promotie-onderzoek uit te voeren. Ivonne Rietjens voor haar enorme steun en begeleiding. Van haar heb ik geleerd wat wetenschap bedrijven is. Wanneer ik een stuk tekst bij jou bracht ter correctie kreeg ik het altijd volledig "gekleurd" terug. Je schreef er dan vaak bij dat ik niet moest schrikken omdat de meeste veranderingen tekstueel waren. In de loop der jaren heb ik er echter wel van geleerd, wat bij het schrijven van dit proefschrift goed te merken was.

Marelle, "mijn" volledige, part-time analiste, voor alle "gevechten" die ik met jou heb uitgevochten. Samen hebben we heel wat eiwitjes gezuiverd waarbij er een echte competitie ontstond voor de hoogste opbrengst en specifieke activiteit. Meer dan drie dagen in de week samen werken met jou was echt te veel. Mijn andere kamergenoten Nicole en Janneke die als collega OIO en BRAIO van Ivonne met dezelfde roodgekleurde artkelen geconfronteerd werden en Jan Willem zonder wiens aanwezigheid ik allang ondergesneeuwd was tussen al die vrouwen. Onze kamer was ongetwijfeld één van de drukste van de vakgroep maar dit stond nooit nuttige gesprekken in de weg.

René, Gijs, Gerlienke, Erik, Arthur en Loeckie die door de keuze van hun afstudeervak allemaal hun bijdrage aan dit proefschrift hebben geleverd. Alle andere studenten van lab 339 (en dat waren er een hele hoop) en de andere labgenoten Ans, Sjaak en Sjef die er ook voor gezorgd hebben dat het op ons lab altijd een gezellige drukte was.

Verder alle andere mensen van de derde en tweede verdieping die mij in de afgelopen vier jaar met raad en daad hebben bijgestaan, Jo Haas en natuurlijk de 496 ratten van het CKP die zich altijd voor '100%' voor mij ingezet hebben.

Familie en vrienden die zich altijd geïnteresseerd hebben getoond en die in mijn vrije uren voor de broodnodige afleiding hebben gezorgd.

Tot slot natuurlijk gaat mijn grootste dank uit naar Angelique die het al die tijd met mij heeft uitgehouden, me heeft gesteund en aangemoedigd bij de tot stand koming van dit proefschrift.

Chapters 2,3,5,6 and 7 have been published and chapter 4 will be published seperately.

Chapter 2

Balvers, W.G., Boersma, M.G., Veeger, C. and Rietjens, I.M.C.M. (1992) Differential cumene hydroperoxide sensitivity of cytochrome P450 enzymes IA1 and IIB1 determined by their way of membrane incorporation. *Biochim.Biophys.Acta* **1117**: 179-187

Chapter 3

Balvers, W.G., Boersma, M.G., Veeger, C. and Rietjens, I.M.C.M. (1993) Kinetics of cytochrome P-450 IA1 and IIB1 in reconstituted systems with dilauroyl- and distearoyl-glycerophosphocholine. *Eur.J.Biochem.* **215**: 373-381

Chapter 4

Balvers, W.G., Boersma, M.G., Veeger, C. and Rietjens, I.M.C.M. The effect of variation in the headgroup and fatty acyl chains of phospholipids on their binding to cytochrome P-450 IIB1.

Chapter 5

Balvers, W.G., Boersma, M.G., Vervoort , J., Ouwehand, A. and Rietjens, I.M.C.M. (1993) A specific interaction between NADPH-cytochrome reductase and phosphatidylserine and phosphatidylinositol. *Eur.J.Biochem.* **218**: 1021-1029

Chapter 6

Balvers, W.G., Boersma, M.G., Vervoort, J. and Rietjens, I.M.C.M. (1992) Experimental and theoretical study on the redox cycling of resorufin by solubilized and membrane bound NADPH-cytochrome reductase. *Chem.Res.Toxicol.* **5**: 268-273

Chapter 7

Boersma, M.G., Balvers, W.G., Boeren, S., Vervoort, J. and Rietjens, I.M.C.M. NADPH-cytochrome reductase catalysed redox cycling of 1,4-benzoquinone; hampered at physiological conditions, initiated at increased pH values. (1994) *Biochem.Pharmacol.* **47**: 1949-1955

Abbreviations

2,3DMBQ	2,3-dimethyl-1,4-benzoquinone
2,5DMBQ	2,5-dimethyl-1,4-benzoquinone
3-MC	3-methylcholanthrene
AROD	alkoxyresorufin O-dealkylation
BQ	1,4-benzoquinone
CHOL	sodium cholate
CuOOH	cumene hydroperoxide
DCBQ	2,3-dichloro-1,4-benzoquinone
DCDCNBQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DOC	sodium deoxycholate
ECOD	ethoxycoumarin O-dealkylation
E_{LUMO}	energy of the lowest unoccupied molecular orbital
EROD	ethoxyresorufin O-dealkylation
E_{SOMO}	energy of the single occupied molecular orbital
$HF_{1e\text{-}red}$	heat of formation of the one electron reduced form
HF_{ox}	heat of formation of the oxidized form
HQ	1,4-hydroquinone
ISF	isosafrole
K_d^r	apparent K_d of the cytochrome P-450 - NADPH-cytochrome reductase complex
K_d^s	apparent K_d of the cytochrome P-450 - substrate complex
K_m^r	apparent K_m for NADPH-cytochrome reductase
K_m^s	apparent K_m for alkoxyresorufins or ethoxycoumarin
LUMO	lowest unoccupied molecular orbital
MBQ	2-methyl-1,4-benzoquinone
MDA	malondialdehyde
Me_2SO	dimethylsulfoxide
NMR	nuclear magnetic resonance
PB	phenobarbital
PROD	pentoxyresorufin O-dealkylation
SDS/PAGE	sodium dodecyl sulphate / polyacrylamide gel electrophoresis
SEM	standard error of the mean
SOMO	single occupied molecular orbital
SQ	1,4-semiquinone

TBA	thiobarbituric acid
TBBQ	tetrabromo-1,4-benzoquinone
TCBQ	tetrachloro-1,4-benzoquinone
TLC	thin layer chromatography
TMBQ	2,3,5,6-tetramethyl-1,4-benzoquinone
triMBQ	2,3,5-trimethyl-1,4-benzoquinone

Phospholipids

DPH-PC	2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine
LPC	L-α-1-acyl-*sn*-glycero-3-phosphocholine
LPE	L-α-1-acyl-*sn*-glycero-3-phosphoethanolamine
LPS	L-α-1-acyl-*sn*-glycero-3-phosphoserine
PA	L-α- diacyl-*sn*-glycero-3-phosphate
PC di10:0	L-α-didecanoyl-*sn*-glycero-3-phosphocholine
PC di12:0	L-α-dilauroyl-*sn*-glycero-3-phosphocholine
PC di14:0	L-α-dimyristoyl-*sn*-glycero-3-phosphocholine
PC di16:0	L-α-dipalmitoyl-*sn*-glycero-3-phosphocholine
PC di18:0	L-α-distearoyl-*sn*-glycero-3-phosphocholine
PC di18:1	L-α-dioleoyl-*sn*-glycero-3-phosphocholine
PC di18:2	L-α-dilinoleoyl-*sn*-glycero-3-phosphocholine
PC di20:4	L-α-diarachidoyl-*sn*-glycero-3-phosphocholine
PC	L-α- diacyl-*sn*-glycero-3-phosphocholine
PE di16:0	L-α-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
PE	L-α- diacyl-*sn*-glycero-3-phosphoethanolamine
PG	L-α- diacyl-*sn*-glycero-3-phosphoglycerol
PI 16:0/18:1	L-α-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoinositol
PI	L-α- diacyl-*sn*-glycero-3-phosphoinositol
PS di16:0	L-α-dipalmitoyl-*sn*-glycero-3-phosphoserine
PS	L-α- diacyl-*sn*-glycero-3-phosphoserine
SM	2-acyl-sphingosine-1-phosphocholine

Contents

Chapter 1

General introduction

A: The cytochrome P450 enzyme system
B: The importance of phospholipids in the cytochrome P450 enzyme system

A1.1 Introduction

Living organisms are exposed to an ever increasing number of *xenobiotics* (i.e. chemicals foreign to the body) such as drugs, food additives, preservatives, pesticides and other agrochemicals and environmental contaminants. The body is equipped with metabolic pathways which can convert these foreign compounds as well as various endogenous substrates like steroids, fatty acids, prostaglandines and ketones to metabolites that can be excluded from the body. In a process called *biotransformation* the water solubility of xenobiotics is increased to make them readily excretable through urine or bile. The biotransformation of foreign chemicals is generally divided into two phases. Phase I metabolism involves the modification of the xenobiotic through oxidation, reduction or hydrolysis. In Phase II metabolism a polar xenobiotic or a product from Phase I metabolism is conjugated with small endogenous molecules (e.g. glucuronic acid, glutathione, sulphate, acetate, glycin etc.). Generally, the biotransformation leads to detoxification of the xenobiotics. However, in some cases the biotransformation can give rise to the formation of reactive intermediates and/or metabolites which are more toxic than the parent molecule (*bioactivation*). These toxic metabolites can cause cell damage.

An important Phase I enzyme system is the cytochrome P450 dependent monooxygenase. Over the last three decades the cytochrome P450 enzyme system has been intensively studied. A complete understanding of the mechanism of action of the cytochrome P450 enzyme system is very important for toxicological research, for example in determining the bioactivation and detoxification of a xenobiotic. But also in the pharmacological and medical field a good knowledge of this enzyme system is of great importance especially because the P450 enzyme system is to a certain extent responsible for the residence time of drugs in the body and thus for the required dosage of medication. Currently pharmaceutical laboratories screen potential drugs on the formation of possible metabolites in *in vitro* systems using the cytochrome P450 enzyme system. Furthermore, the use of the P450 enzyme system has also been suggested in organic chemistry, especially in the synthesis of stereo- and / or regio selective chemicals, and in the battle against the ever increasing environmental contamination [1,2].

The following paragraphs describe in more detail important general features of the cytochrome P450 enzyme system like cytochrome P450 multiplicity and inducibility, the structure of the individual protein constituents of the enzyme system and the catalytic cycle.

A1.2 Occurence and cellular location of the cytochrome P450 enzyme system

The cytochromes P450 enzyme system occurs in biological systems as diverse as microorganisms, plants, animals and man. Of all mammalian tissues, the liver is the organ containing the highest P450 amounts, although cytochromes P450 are also found in the lungs, skin, kidneys, nose, spleen, testes, intestines and other tissues but in relatively low amounts [3-6].

The mammalian cytochrome P450 enzyme system is associated with the membrane of the smooth endoplasmatic reticulum. The enzyme system belongs to the class of oxygen activating enzymes that catalyse biological oxidations by transferring electrons from NADPH to molecular oxygen. The enzyme system is a multi-enzyme complex composed of (i) *cytochromes P450*, hemoproteins that catalyse the final electron transfer to molecular oxygen and the insertion of an oxygen atom into the substrate, (ii) *NADPH-cytochrome reductase*, a flavoprotein that transfers electrons from NADPH to cytochrome P450 or cytochrome b_5, (iii) *cytochrome* b_5, a hemoprotein that takes part in the transfer of electrons to cytochrome P450 for some substrate conversions [7] and (iv) *NADH-cytochrome reductase*, a flavoprotein that tranfers electrons from NADH to cytochrome b_5. A schematic presentation of the electron flow through the mammalian cytochrome P450 enzyme system from the endoplasmatic reticulum is presented in Figure A1.1.

For mitochondrial and bacterial cytochromes P450 the electrons are transferred from NADH via a flavoprotein (for example adrenodoxin reductase for mitochondrial cytochrome P450 and putidaredoxin reductase for bacterial cytochrome P450 from *Pseudomonas putida*) and a ferredoxin (an iron-sulfur ([2Fe-2S]) protein; adrenodoxin and putidaredoxin for respectively mitochondrial and bacterial cytochrome P450) to molecular oxygen [8,9].

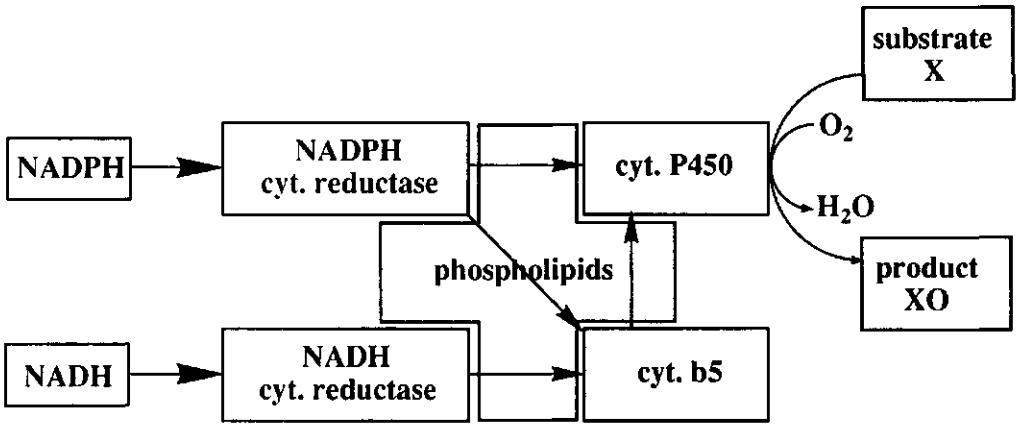


Figure A1.1: Electron flow through the mammalian cytochrome P450 enzyme system

A1.3 Cytochrome P450 multiplicity

One of the most characteristic features of the P450 enzyme system is its ability to metabolise in addition to endogenous substrates like steroids, fatty acids, prostaglandines and ketones, an almost unlimited number of biologically occurring and xenobiotic substances (currently estimated to be more than 250,000 different compounds) [10-14]. This characteristic originates from the existence of multiple cytochrome P450 enzymes. Upto now, more than 150 different cytochrome P450 forms have been characterised [2]. In rats at least 38 different P450 enzymes were described [1]. When the existence of multiple cytochrome P450 forms was first demonstrated [15] it was not known whether different species and tissues would have similar forms. Research, done in the seventies and early eighties, on different forms of cytochrome P450 resulted in a confusing variety of trivial names that were based on, for example, the animal from which the cytochrome P450 form was isolated, chemicals that were used to pretreate the animal prior to isolation of the cytochrome P450 form, a substrate metabolised by the P450 form or a specific reaction catalysed by the isolated cytochrome P450 form. In some cases numbers or letters were assigned in series. Nebert *et al.* [16,17] introduced a systematic nomenclature in which the individual P450 proteins were devided into families and subfamilies depending on the

alignment and percent of similarity of the nucleotide sequences of their genes. Those P450 proteins from all sources with 40% or greater sequence identity are included in the same family, presented by a Roman number, and those with greater than 55% identity are then included in the same subfamily, presented by a capital letter. The individual genes are then arbitrarily given an additional Arabic number. This nomenclature is now generally accepted and examples for some different forms of rat cytochrome P450 together with their trivial names as presented in earlier studies are presented in Table A1.1.

Table A1.1: Example of different nomenclatures of some different forms of rat cytochrome P450

nomenclature Nebert [16,17]	nomenclature Guengerich [12,18]	nomenclature Ryan [12]	nomenclature Waxman [12]	nomenclature Schenkman [12]
IA1	βNF-B	c	-	-
IA2	ISF-G	d	-	-
IIA1	UT-F	a	PB-3	RLM2b
IIB1	PB-B	b	PB-4	PBRLM5
IIB2	PB-D	e	PB-5	PBRLM6
IIC6	PB-C	k	PB-1	PBRLM4
IIC7	-	f		RLM5b
IIC11	UT-A	h	2c	RLM5
IIC12	UT-I	i	2d	fRLM4
IIC13	-	g	-	RLM3
IIE1	-	j		RLM6
IIIA1	PCN-E	p	PB-2a	-

A1.4 Cytochrome P450 induction

The level of many forms of cytochrome P450 in the different organs of the body can be increased by exposure to a chemical agent. So far 3 major classes of cytochrome P450 inducers are known which increase the level of different families of P450 forms; (i) the phenobarbital type, inducing families IIB and to a minor extent IIC, (ii) the 3-methylcholanthrene type (polycyclic, aromatic hydrocarbons), inducing families IA and to a minor extent IIA and (iii) the steroid type (for

example pregnenolone-16α-carbonitrile (PCN)), inducing family IIIA [18]. In addition to these three major classes, two minor classes of inducible P450 forms have been identified; the ethanol-inducible P-450's (family IIE) and clofibrate-inducible P-450's (family IVA) [18].

The mechanism by which inducers elevate the level of specific cytochrome P450 forms is not yet fully understood but it is believed to be different for individual forms of cytochrome P450. The induction of cytochrome P450 IA by polycyclic aromatic hydrocarbons is supposed to involve a cytoplasmatic Ah (Aryl hydrocarbon) receptor [19-21]. After binding to the receptor, the inducer-receptor complex (AhR) is translocated to the nucleus. In the ligand-free form the receptor is bound to a 90 kDa "Heat shock Protein" (HsP90) similar to steroid hormon receptors [22]. Furthermore, the protein complex is believed to contain a metalion, probably molybdenum. Upon binding the inducer, the receptor is activated. During the process of receptor activation, HsP90 dissociates from the inducer-receptor complex thereby liberating the DNA binding domain on the receptor [23]. Recently it was demonstrated that another protein is involved in the translocation of the activated AhR to the nucleus, namely the Ah receptor nuclear translocator (Arnt) [24]. The interaction of the AhR protein with DNA involves socalled "zinkfingers" in the DNA binding site [25] which make contact with the "major groove" of the DNA. The binding stimulates the transcription of the P450 IA gene. The transport of mRNA from the nucleus to the cytosol, which is suggested to be the rate-limitting step of biosynthesis of the protein [26], then results in the selective biosynthesis of the enzyme into which mitochondrially derived heme is inserted to form the holoenzyme. Isosafrole and other methylenedioxyphenyl inducers which are also capable of inducing cytochrome P450 IA do not appear to act via the Ah receptor [27]. From computer graphic studies of the molecular dimensions and electronic structures of inducers of cytochrome P450 IA and specific substrates and inhibitors of cytochrome P450 IA, the conformation of the cytosolic receptor was demonstrated to resemble that of the active site of the enzyme [28]. For further information on cytochrome P450 IA regulation the reader is referred to papers of Fujii-Kuriyama *et al.* [29] and Bresnick and Houser [30].

Phenobarbital induction of cytochrome P450 IIB is suggested to result from increased levels of P450 IIB mRNA [31,32] resulting from increased transcription of the corresponding P450 IIB gene. Recognition

of the inducer is suggested to be mediated by a phenobarbital type receptor [33,34] although receptor-independent mechanisms have also been postulated [32].

The induction of cytochrome P450 IIE proceeds primarily by stabilization of the protein and its mRNA [35,36]. Binding of the substrate (for example ethanol or acetone) or its metabolite has been suggested to protect the P450 IIE protein from rapid degradation.

The mechanisms of induction of cytochrome P450 IIIA were shown to be diverse. Induction by pregnenolone-16α-carbonitrile (PCN) and dexamethasone was demonstrated to result from an increase in P450 III mRNA [37,38] whereas endogenous glucocortecoid mediated induction of this enzyme involves a cytosolic receptor [39].

The induction of cytochrome P450 IVA by clofibrate has been reported to be mediated by a receptor, activated by a peroxisome proliferating compound [40,41]. Upon clofibrate administration the gene transcription is suggested to increase rapidly, followed by an elevated cytochrome P450 IVA mRNA and protein level [42].

A1.5 Structure of the various protein constituents of the cytochrome P450 enzyme system

In the following paragraph the structure of the individual protein components of the enzyme system will be discussed in more detail. Special attention will be given to structural aspects that might be of importance for membrane incorporation and protein-protein interactions in the P450 system.

A1.5.1 Cytochrome P450

Currently, the only direct method for the determination of the three-dimensional structure of high molecular weight proteins like the cytochromes P450 (± 50 kDa) is through high-resolution X-ray crystallography. For the bacterial, camphor metabolising, soluble cytochrome P450 CAM from *Pseudomonas putida* a three dimensional structure, obtained from high-resolution X-ray crystallography, has been reported [43] and more recently, the three dimensional structure of another bacterial cytochrome P450, P450 BM-3 from *Bacillus megaterium*, was elucidated [44]. In contrast, the information on the three

dimensional structure of mammalian cytochromes P450 (50 kDa) is limited because these proteins have not (yet) been succesfully crystalysed. This is mainly due to the fact that, unlike cytochrome P450 CAM, mammalian cytochromes P450 are membrane bound resulting in protein preparations containing disturbing surfactants and phospholipids and / or irregular protein aggregates.

Several models for the incorporation of vertebrate cytochrome P450 into the membrane have been proposed over the last decade. Early models suggested cytochrome P450 to be an intrinsic membrane protein, deeply embedded in the phospholipid bilayer with multiple α-helices spanning the membrane [45-49]. Recent experiments using site-specific antibodies and chimeric proteins however, demonstrated this model to be unlikely and a new model on the topology of membrane bound cytochrome P450 was proposed with an anchoring domain of one [50-59] maybe two [60-63] membrane spanning helices at the N-terminus and a large hydrophilic domain exposed to the cytosol which may have one or two additional peripheral membrane contacts [62, 64,65]. Based on gene analogy of mammalian and bacterial cytochrome P-450 the hydrophilic domain is suggested to resemble the overall shape of P450 CAM; a triangular prism, 30 Å thick, with sides of 55 to 60 Å [66]. This domain contains the heme group and the binding sites for NADPH-cytochrome reductase and cytochrome b_5. Lysine residues located on the surface of the hydrophilic domain of the cytochromes P450 are suggested to play an important role in the interaction of the cytochromes P450 with NADPH-cytochrome reductase and cytochrome b_5 [67-69]. A model presenting membrane associated cytochrome P450 with one membrane spanning helix is presented in Figure A1.2. The heme orientation of cytochrome P450 in relation to the plane of the membrane is at present not conclusively established. The opinions on this subject vary from a parallel orientation [61] to a perpendicular orientation with respect to the plane of the membrane [57].

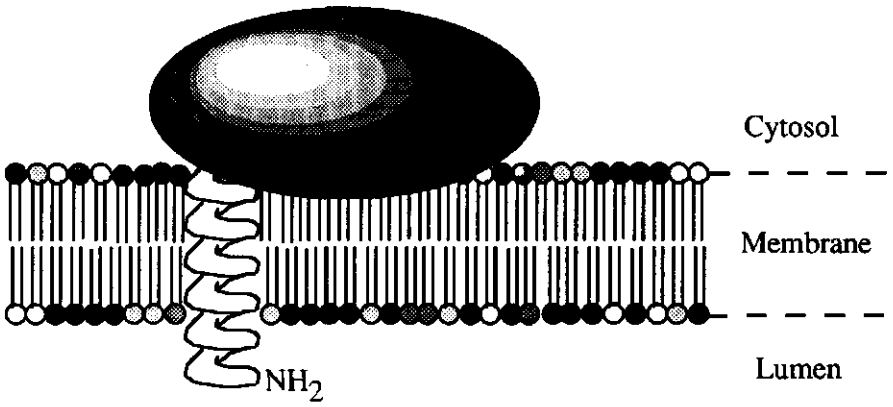


Figure A1.2: Membrane topology of mammalian cytochrome P450

A1.5.2 NADPH-cytochrome reductase

NADPH-cytochrome reductase is a membrane bound flavoprotein and the primary electron donor for the cytochrome P450 enzymes although it is also capable of reducing cytochromes c and b_5. In addition NADPH-cytochrome reductase is capable of reducing artificial electron acceptors such as ferricyanide, 2,6-dichlorophenolindophenol and a great number of low molecular weight compounds with suitable redox potentials such as, for example, quinones [70]. The protein has a molecular mass of about 76 kDa and is anchored to the membrane by a membrane spanning binding domain of 6-10 kDa at the acetylated N-terminus [71]. Without this membrane binding domain the protein is unable to reduce cytochrome P450 [72,73]. The large hydrophilic C-terminal domain is exposed to the cytosol.

The enzyme contains two prosthetic groups per polypeptide chain; one FAD and one FMN [74] which are responsible for the electron transport to cytochrome P450. The midpoint potentials of the two flavin molecules are presented in Table A1.2 [75,76].

Table A1.2: Redox potentials of the different redox couples in NADPH-cytochrome reductase

redox couple	redox potential (mV)
$FAD \Leftrightarrow FADH^{\bullet}$	-290
$FADH^{\bullet} \Leftrightarrow FADH_2$	-365
$FMN \Leftrightarrow FMNH^{\bullet}$	-110
$FMNH^{\bullet} \Leftrightarrow FMNH_2$	-270

Based on a study using NADPH-cytochrome reductase which was free of FMN the following sequence of electron transfer in the cytochrome P450 enzyme system was proposed [77];

$$\text{NADPH} \Rightarrow \text{FAD} \Rightarrow \text{FMN} \Rightarrow \text{Cytochrome P450}$$

The low redox potential of FAD enables it to accept two electrons from NADPH. A sulfhydryl group and an ε-amino group (perhaps of lysine) [78] located in the vicinity of the NADPH binding site are suggested to be involved in the interaction with NADPH. Upon accepting the electrons from NADPH, FAD transports the electrons to FMN which in its turn, when fully reduced, can transfer the electrons, one at a time, to cytochrome P450. Adjacent to the FMN binding region there is a cluster of 6 carboxyl groups which are part of the possible site of electrostatic interaction with cytochromes *c* and P450 [79,80]. Selective methylamidation of these carboxyl groups suppresses the ability of NADPH-cytochrome reductase to reduce cytochromes P450 and *c* but stimulates the reduction of cytochrome b_5 by NADPH-cytochrome reductase [80] indicating that the mechanism of interaction of reductase with cytochromes P450 and *c* is different from that with cytochrome b_5.

A1.5.3 Cytochrome b_5

Cytochrome b_5 is an amphipatic, heme containing enzyme of 16 kDa and composed of at least two domains. Proteolytic treatment leads to a heme containing, hydrophilic catalytically active fragment with a molecular weight of about 11 kDa. This catalytically active part was obtained in crystalline form and its structure was determined by X-ray analysis and NMR [81,82] as a spherical ellipsoid with a diameter of 31 Å and a height of 38 Å. The heme group has two histidines as axial ligands and two propionate residues from the heme are exposed to the water phase. Carboxyl residues located on the surface of the catalytic domain of cytochrome b_5, together with the propionate groups participate directly in the interaction with NADPH-cytochrome reductase and NADH-cytochrome reductase [83,84].

The hydrophilic part is connected to a hydrophobic, membrane incorporated part by a flexible region of about 10 amino acids which allows a high mobility of the hydrophilic part [85]. Probably due to this flexible region the heme group of cytochrome b_5 has no definite orientation with respect to the plane of the membrane [86]. The membrane fragment is a C-terminal fragment of about 40 amino acids

[85]. Together with the carboxyl residues on the hydrophilic domain, the hydrophobic part is essential for interaction with the other proteins of the enzyme system.

A1.5.4 NADH-cytochrome reductase

The primary electron donor of cytochrome b_5 is NADH-cytochrome reductase. It is a membrane bound, flavin (FAD) containing protein of about 33 kDa with the active site located at the cytosolic side of the endoplasmatic reticulum membrane [66]. The redox potential of this flavin containing protein was determined to be -280 mV [87] and it can use both NADH and NADPH as electron source although the latter is actually used much less frequent. The hydrophilic and hydrophobic parts are, like in cytochrome b_5, assumed to be connected by a short, extremely flexible region [88] which enables the enzyme to serve as an electron carrier to various acceptors. The membrane binding domain is a strongly hydrophobic N-terminal fragment. The amino-terminal glycine is acylated by myristic acid [89].

A1.6 The cytochrome P450 catalytic cycle

The cytochrome P450 enzyme system is capable of catalysing a variety of reactions, including oxidative and reductive dehalogenation, oxidative deamination, aliphatic-, aromatic- and N-hydroxylation, N-oxidation, epoxidation and O-, N- and S-dealkylation. Most of these reactions are so called monooxygenase reactions in which one of the atoms of molecular oxygen is transfered to the substrate and the other atom is incorporated in a water molecule.

$$R + O_2 + 2e^- + 2H^+ \Rightarrow RO + H_2O$$

The catalytic mechanism of the cytochrome P450 dependent monooxygenase reaction is depicted in Figure A1.3. The individual steps in the catalytic cycle are discussed in detail hereafter.

A1.6.1 Cytochrome P450 Fe^{3+}

The catalytic site of the enzyme system contains an Fe^{3+}-protoporphyrin IX as the prosthetic group [90-92], whose ferrous-carbon monoxide complex exhibits a characteristic light absorption peak around 450 nm.

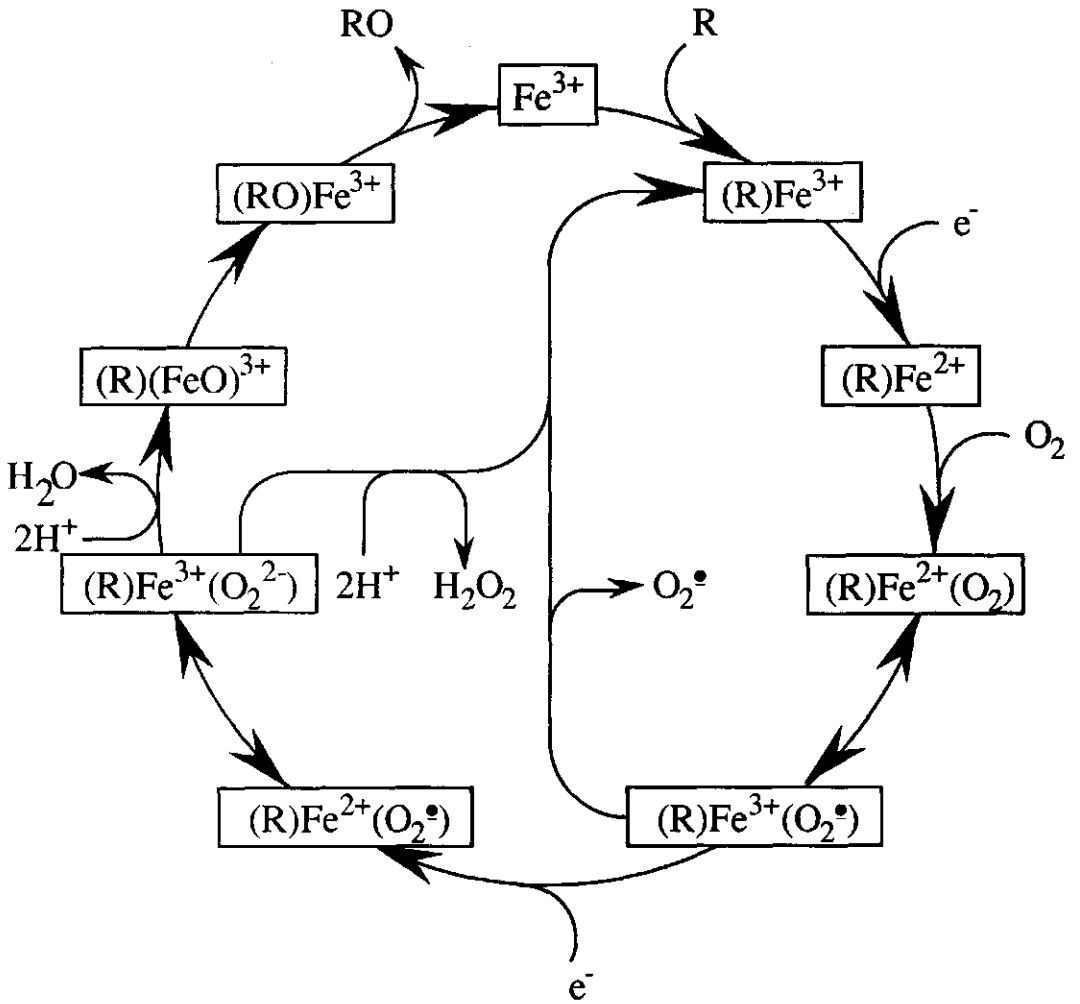


Figure A1.3: Catalytic mechanism of the cytochrome P450 monooxygenase reaction

The iron atom of the ironporphyrin functions as an Fe^{2+}/Fe^{3+} redox couple in the catalytic cycle. The four N-atoms from the porphyrin molecule act as the planar ligands of the Fe^{3+}. The fifth ligand of the iron heme is a cysteinyl thiolate group [93] which in the bacterial cytochrome P450 CAM originates from Cys 357 [43]. This axial cysteine ligand contributes to the specific function of cytochrome P450 compared to oxygen binding hemoproteins like myoglobin and hemoglobin, in which the fifth coordinate position is occupied by a histidine residue. The typical light absorption peak at 450 nm also originates from this axial

cysteine ligand. Denaturation of the protein shifts this absorption band from 450 to 420 nm as a result of either a distorted iron-thiolate bond [94] or a replacement of the thiolate by another ligand [95]. It has been suggested that in the denatured state an imidazole is coordinated to the iron instead of a thiolate [96]. The other axial ligand of cytochrome P450 might be an OH-containing group - probably originating from tyrosine or a water molecule - resulting in the hexacoordinated, low spin (S=1/2) Fe^{3+} form with the iron in the plane of the porphyrin ring. However, the sixth ligand position can also remain unoccupied, resulting in the pentacoordinated, high spin (S=5/2) Fe^{3+} form, with the iron atom slightly lifted out of the plane of the porphyrin molecule (Figure A1.4). The high and low spin conformers of the heme group are in equilibrium with each other and the position of this equilibrium is dependent on the cytochrome P450 form, the temperature and the ionic strength of the solvent [97].

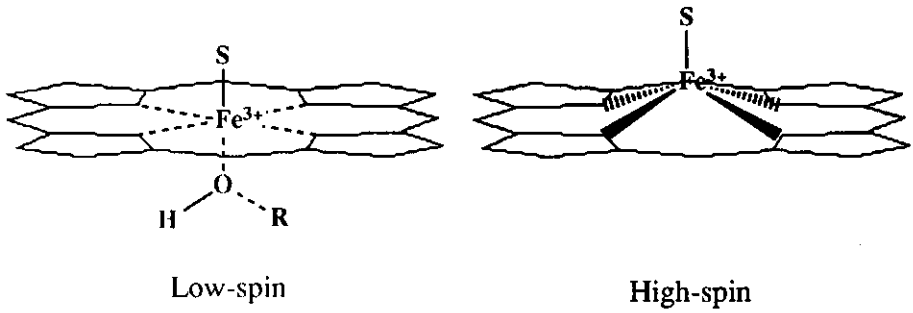


Figure A1.4: Low and high spin form of cytochrome P450

A1.6.2 Cytochrome P450 Fe^{3+} + R ⇒ cytochrome P450 Fe^{3+}(R)

The binding of substrate to cytochrome P450 is generally accompanied by a shift in the spin equilibrium. This change in the spin equilibrium results in characteristic spectral changes of the heme chromophore. The spectral changes can be divided into three categories. The majority of the substrates (mainly hydrophobic substrates) induce a difference spectrum which is characterised by a peak at 387 nm and a trough at 421 nm. This is denoted as a type I difference spectrum and explained as a shift in the spin equilibrium from low-spin to high-spin cytochrome P450 (Figure A1.5). Type I difference spectra have also been observed upon addition to cytochromes P450 of NADPH-cytochrome reductase [98], phospholipids [98-100], cytochrome b_5 [101-104] and free fatty acids [105]. Other substrates (for example alcohols and ketones) produce a reverse type I

difference spectrum with a peak at ±420 nm and a trough at 385-390 nm. This is interpreted as a shift towards the low-spin conformer. Finally a group of substrates (nitrogen containing compounds) induce a type II difference spectrum with a maximum between 425 and 445 nm and a minimum between 390 and 420 nm. It is assumed that type I substrates (mainly hydrophobic molecules) bind to a hydrophobic site near the heme group of cytochrome P450, while type II substrates occupy the sixth axial ligand position [106-107].

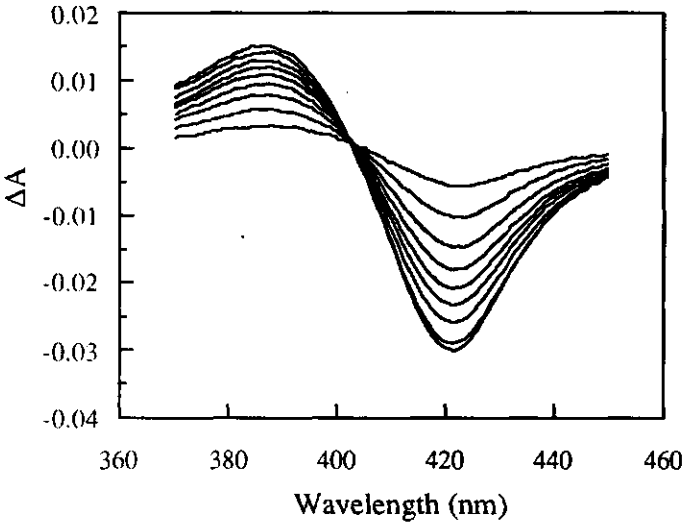


Figure A1.5: Type I difference spectra of cytochrome P450 IIB1 in the presence of increasing concentrations of ethoxycoumarin

The substrate binding site has been reported to be facing (or partly buried in) the membrane [49,108], although this is contradicted by others [57]. Because of the lipophilic character of most substrates, the active sites of the cytochromes P450 are assumed to be hydrophobic domains located near the heme [109-111]. Experiments involving the chemical modification of tyrosines suggest that Tyr 380 is located in the active site of cytochrome P450 LM_2 (cytochrome P450 IIB1 new nomenclature) and involved in substrate binding together with Phe 377 [45,112].

For some cytochrome P450 enzymes a change in the spin equilibrium in favor of the high spin state results in a significant increase in the redox potential. For instance, upon camphor binding, the redox

potential of bacterial cytochrome P450 CAM has been reported to increase from -303 mV to -173 mV [97,113] which would facilitate its reduction. Although an increase in the redox potential upon substrate binding was not observed for other P450 enzymes [114] it is generally assumed that substrate binding can facilitate the reduction of cytochrome P450.

A1.6.3 Cytochrome P450 Fe^{3+}(R) + e^- ⇒ cytochrome P450 Fe^{2+}(R)

In the next step of the reaction cycle cytochrome P450 becomes one-electron reduced. For mitochondrial and bacterial cytochromes P450, the electron comes from NADH via a flavoprotein and a ferredoxin (an [2Fe-2S]-protein) [8,9] whereas for mammalian cytochrome P450 systems the first electron for the reaction comes from NADPH via NADPH-cytochrome reductase.

Because pyridine nucleotides are two electron donors and cytochrome P450 can only accept one electron at a time, NADPH-cytochrome reductase must be a two electron acceptor and a one electron donor. This means that during the electron transfer process the reductase must be able to store a radical electron in a form which is insensitive to molecular oxygen. This oxygen stable semiquinone form is most likely FAD-FMNH• [76]. The exact mechanism by which electrons are transported from NADPH to cytochrome P450 is at present still unclear but a possible mechanism was suggested by Iyanagi *et al.* [76] (Figure A1.6). In a "priming" reaction the low potential flavin, FAD, accepts two electrons from NADPH [115]. Through intramolecular transfer the electrons are transported to the high potential flavin, FMN. The FAD-$FMNH_2$ then transfers one electron to cytochrome P450, resulting in the formation of the oxygen stable semiquinone form, FAD/FMNH•. This semiquinone form is rapidly reduced by NADPH to the three electron reduced form, $FADH_2$-FMNH•. Through intramolecular electron transfer an electron is again transported to FMN, leading to the formation of FAD•-$FMNH_2$, which also can donate an electron to cytochrome P450. The remaining FAD•-FMN• is converted through intramolecular electron transfer to FAD-$FMNH_2$ which can enter a new cycle.

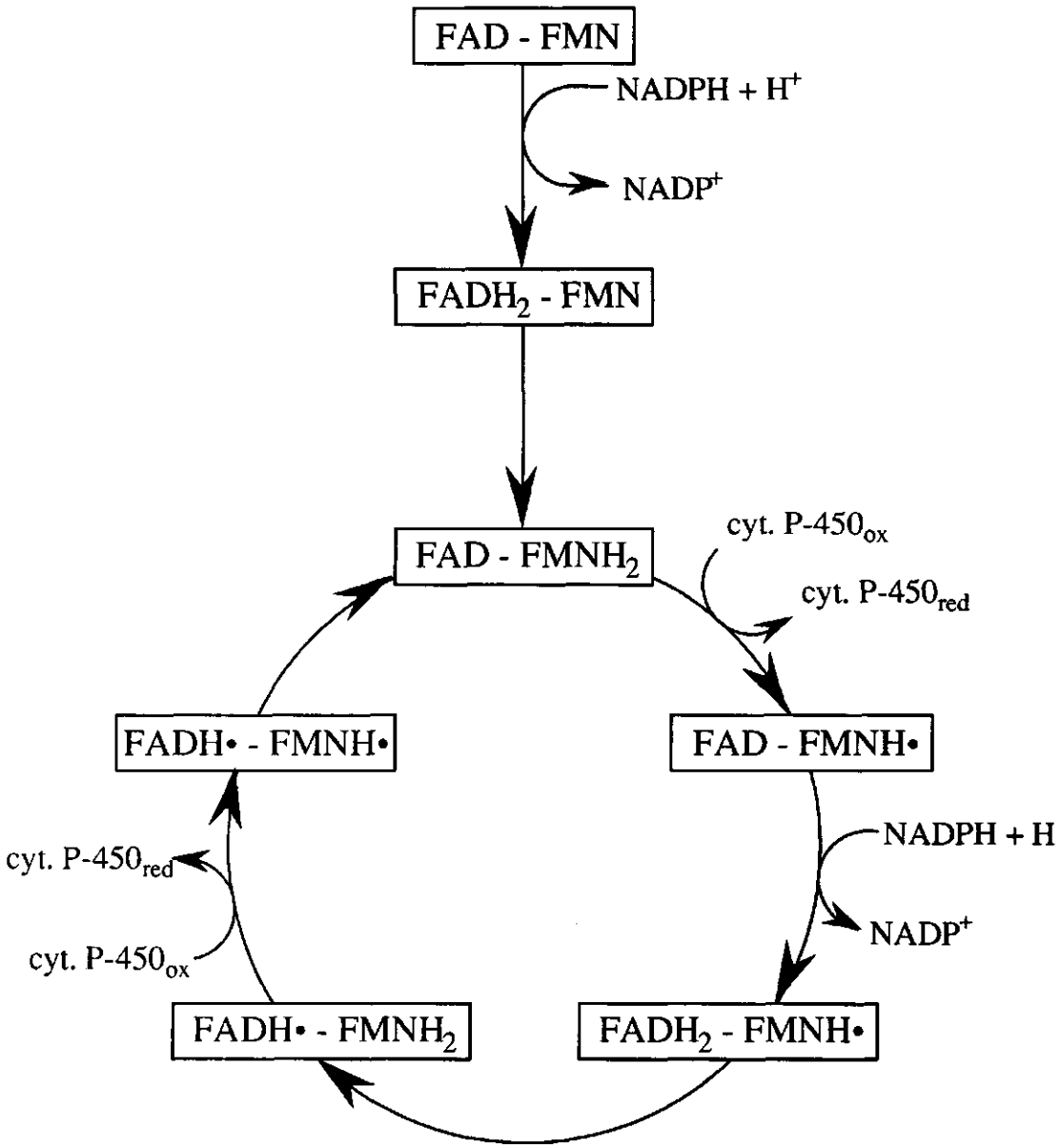


Figure A1.6: The catalytic cycle of NADPH-cytochrome reductase

Stopped flow studies with purified proteins have provided evidence for the fact that the reduction of cytochrome P450 in the first electron transfer process is a biphasic reaction, composed of two independent first-order partial reactions [116]. Since the majority of the cytochromes P450 is reduced in the rapid partial phase, this one is considered to be the

physiologically relevant process [97]. Several reaction models were proposed for the reduction of cytochrome P450 by NADPH-cytochrome reductase.

1. The first model is the "statistical collision model" in which both cytochrome P450 and NADPH-cytochrome reductase are randomly distributed over the membrane. The rate of reduction is supposed to be determined by the lateral mobility of the proteins in the membrane. This model, however, does not explain the biphasic character of the reduction of cytochrome P450 [117-121].
2. In the so-called "sequential spin state model", the high- and low-spin form of the cytochrome P450 are supposed to exhibit specific reactivities. Reduction of the high-spin fraction is suggested to be related to the rapid partial reaction, whereas the reduction of the low-spin fraction is supposed to represent the slow partial reaction. The slow reduction of the low-spin fraction is supposed to occur via the rate limiting relaxation into the high-spin conformation [122-124]. However, the relaxation of the spin equilibrium was demonstrated to proceed in the nanosecond region [125,126], thereby excluding rate limitation by this step. As an alternative an additional conformational change, being spectrally invisible, has been proposed to represent the rate limiting step responsible for the slow reduction phase [124].
3. In the "redox state control model" cytochrome P450 reduction is suggested to occur by the biphasic kinetics due to its reduction by NADPH-cytochrome reductase with different redox states [127]. This model is based on a dependency of the phase distribution of the reduction process on the NADPH concentration, but it is questioned because of the occurence of the biphasic character of the reduction process even under conditions of high NADPH and reductase concentration.
4. The fourth model, the so-called "cluster model" is based on the high ratio of cytochrome P450 to reductase (15 to 30) in the endoplasmatic reticulum membrane [128]. According to this model, the reductase is surrounded by a cluster of cytochrome P450 molecules. This organisation implies a sweet contact between the cytochrome P450 and the reductase enabling efficient electron transfer. Therefore, reduction of the cytochromes P450 in this cluster is proposed to correspond to the fast partial reduction. The slow partial reduction would correspond to

the reduction of non-associated cytochrome P450 molecules after a rate-limiting exchange with clustered cytochromes P450 [129-132].

A1.6.4 Cytochrome P450 $Fe^{2+}(R) + O_2 \Rightarrow$ cytochrome P450 $Fe^{2+}(O_2)(R) \Leftrightarrow Fe^{3+}(O_2^{\bullet})(R)$

After the first one electron reduction oxygen binds to the enzyme, occupying the sixth coördinate, thus forming the hexacoördinated $Fe^{2+}(O_2)(RH)$. Through electron transfer from Fe^{2+} to O_2 the $Fe^{3+}(O_2^{\bullet})(RH)$ is formed [8]. At this stage the cycle can derail by the release of a superoxide anion and the return of the enzyme to the $Fe^{3+}(RH)$ state. The superoxide anion is reactive and might inactivate cytochrome P450 by oxidizing the heme group [133-137]. Furthermore, the superoxide anion can undergo an iron-catalysed Haber-Weiss reaction

$$O_2^{\bullet} + H_2O_2 \xRightarrow{Fe^{2+/3+}} OH^- + OH^{\bullet} + O_2$$

yielding the highly reactive hydroxyl radical (HO•). This hydroxyl radical may inactivate P-450 and other proteins and / or initiate peroxidation by hydrogen abstraction from a polyunsaturated fatty acid of a membrane phospholipid [138].

A1.6.5 Cytochrome P450 $Fe^{3+}(O_2^{\bullet})(R) + e^- \Rightarrow$ cytochrome P450 $Fe^{2+}(O_2^{\bullet})(R) \Leftrightarrow Fe^{3+}(O_2^{2-})(R)$

A major difference between the introduction of the first and second electron is that the first electron is donated almost exclusively by NADPH via NADPH-cytochrome reductase, whereas the second electron can be donated by both NADPH via NADPH-cytochrome reductase and NADH via NADH-cytochrome reductase and cytochrome b_5 [9,139]. A contribution of the cytochrome b_5 electron donating system to the second electron reduction process is, however, not observed for all substrates [7,140,141]. For mitochondrial and bacterial cytochromes P450 the first and second electron are both donated by the NADH-flavoprotein-ferredoxin system [8].

After the second electron reduction the catalytic cycle can derail, namely by the release of hydrogen peroxide from which in a Haber-Weiss reaction - as indicated above - hydroxyl radicals are formed which might inactivate cytochrome P450 and initiate lipid peroxidation [138].

Although the exact electron density distribution within the 2-electron reduced oxygen bound P-450 complex is not yet known, it is

suggested that a second electron transfer to molecular oxygen occurs followed by protonation of the terminal oxygen atom resulting in formation of a $Fe^{3+}(O_2H^-)$ intermediate. This process is supposed to facilitate the next step in the catalytic cycle [142].

A1.6.6 Cytochrome P450 $Fe^{3+}(O_2^{2-})(R) + 2H^+ \Rightarrow$ cytochrome P450 $(FeO)^{3+}(R) + H_2O$

It is generally assumed that the heterolytic cleavage of the O-O bond in the $Fe^{3+}(O_2^{2-})R$ intermediate results in the formation of a high valency iron-oxo complex. The exact distribution of the electrons in this reactive intermediate is still under debate but all hypotheses include the removal of two electrons from the iron (forming Fe^{5+}) by oxygen (forming O^{2-}) resulting in a high valency iron-oxo center with a formal charge of 3^+. Possible distributions of the electrons in this intermediate might be:

- $Fe^{5+}(O_2^{2-})$ [142-145], with an iron that has a valency of five (perferryl). However, upto now such a perferryl iron has not yet been observed in any hemoprotein.
- $Fe^{4+}(O_2^{\bullet})$ [142,143,146]. This distribution explains the radical character of the iron-oxo species, which is in accordance with the radical mechanism suggested for several cytochrome P450 dependent biotransformations (see below).
- $(protein/porphyrin)^+(Fe^{4+})(O_2^{2-})$ with a porphyrin or protein centered radical because the iron-oxo species is suggested to have extracted an electron from the porphyrin ring or (perhaps) from the protein [142,146]. Although the role of the protein or the porphyrin in the iron-oxo complex remains to be proven, the porphyrin ring, protein chain and / or cysteine ligand might be important in the reactive intermediate by stabilizing the highly electrophilic iron-oxo species by (partial) electron donation.

A1.6.7 Cytochrome P450 $(FeO)^{3+}(R) \Rightarrow$ cytochrome P450 $Fe^{3+}(RO)$

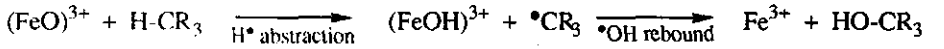
In this step of the catalytic cycle the substrate is converted to product by transfer of the oxygen atom from the $(FeO)^{3+}$ species to the substrate. This results in, for example, alifatic- or aromatic hydroxylation, epoxidation or heteroatom oxidation. Some generally accepted mechanisms and some hypotheses for cytochrome P450$(FeO)^{3+}$ catalysed substrate conversions are presented in Figure A1.7 [142-150]

After release of the product the ferric form of the enzyme is ready to enter the next reaction cycle.

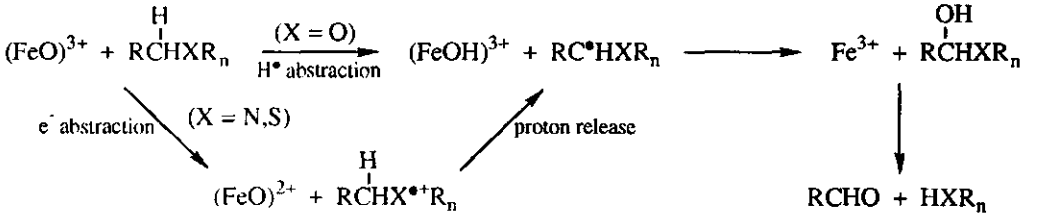
A1.7 An additional component of the cytochrome P-450 enzyme system

In this thesis so far, the properties of the individual protein constituents of the enzyme system and their role in cytochrome P450 substrate conversions have been discussed. However, after the first succesful reconstitution of the cytochrome P450 enzyme system in 1968 it was demonstrated that in addition to cytochrome P450, NADPH-cytochrome reductase, cytochrome b_5 and NADH-cytochrome reductase a fifth, non-protein component was also of importance to generate a reconstituted cytochrome P450 enzyme system with optimal catalytic activity. This fifth component, later demonstrated to be the phospholipids of the endoplasmatic reticulum membrane, has been shown to influence cytochrome P450 dependent activities. The role of phospholipids in the cytochrome P450 enzyme system and the interactions of phospholipids with cytochrome P450 and NADPH-cytochrome reductase will be discussed in more detail in Chapter 1B.

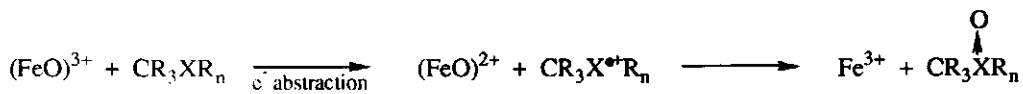
Aliphatic hydrocarbon hydroxylation



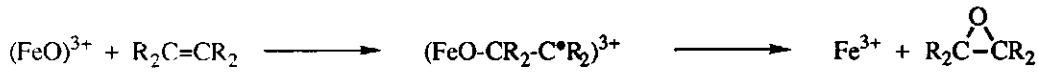
Heteroatom dealkylation (X = O,N,S)



Heteroatom oxygenation (X = N,S,I,P)



Alkene or arene epoxidation



Aromatic ring hydroxylation

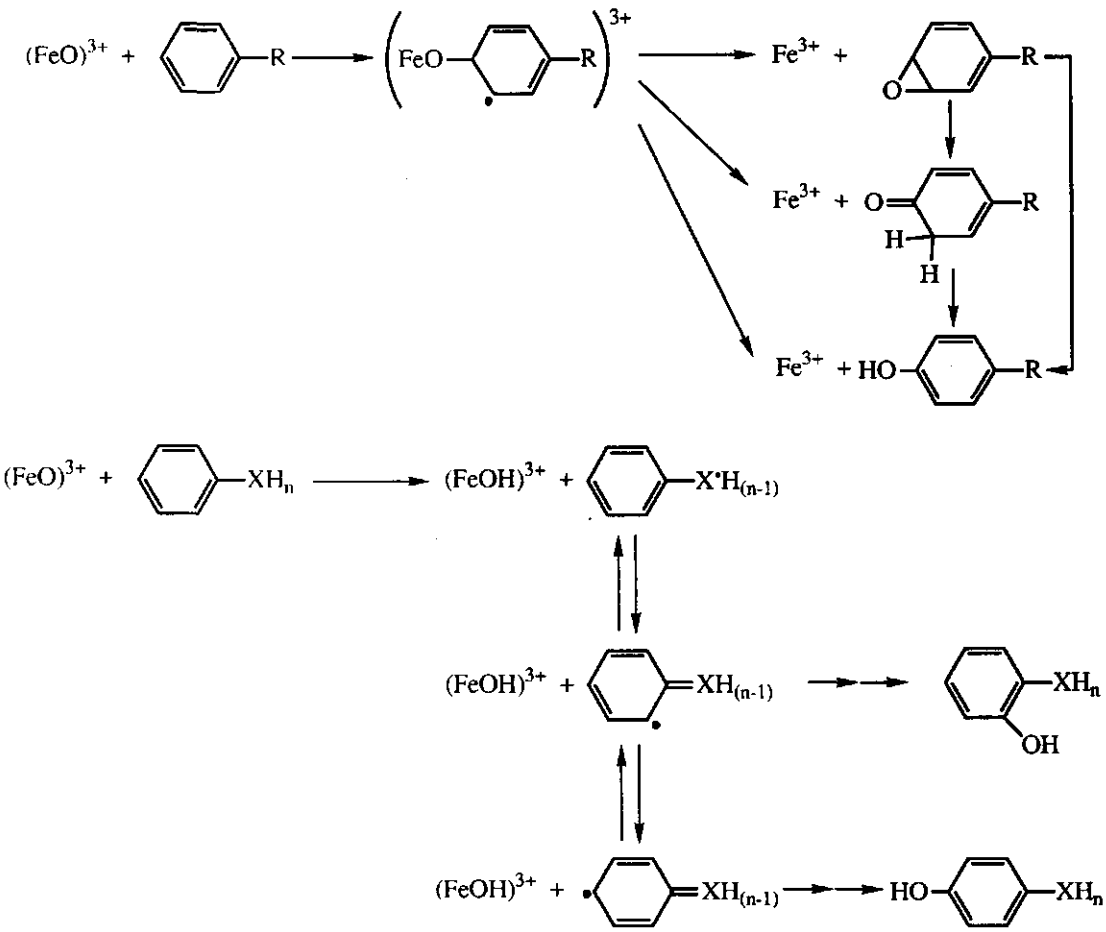


Figure A1.7: Possible mechanisms for cytochrome P450 $(FeO)^{3+}$ catalysed substrate conversions

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General introduction

A: The cytochrome P450 enzyme system

B: The importance of phospholipids in the cytochrome P450 enzyme system

B1.1 Introduction

The properties of membrane-bound enzyme systems which are associated with the endoplasmatic reticulum membrane, like the cytochrome P450 system, may be influenced by characteristics of the membrane. Among the membrane characteristics that have been shown to influence the cytochrome P450 enzyme system are membrane fluidity (determining the lateral and rotational mobilities of the various protein constituents of the cytochrome P450 system), membrane charge (influencing electrostatic protein-protein and lipid-protein interactions) and the hydrophobic character of the core of the membrane (contributing to substrate accumulation in the vicinity of the enzymes) [1-10] These physical properties of the membrane are largely determined by the membrane phospholipids, important constituents of the membrane. This chapter focusses on the structural features of phospholipids and of the endoplasmatic reticulum membrane and especially on the effects of phospholipids on the cytochrome P450 enzyme system.

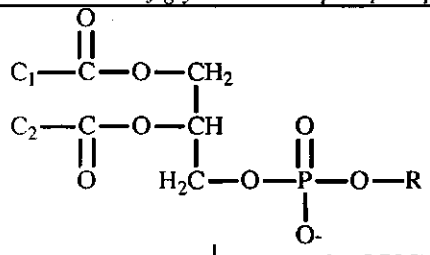
B1.2 General structure of membrane phospholipids

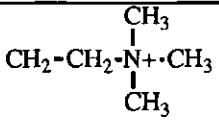
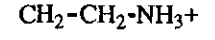
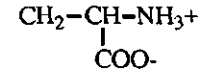
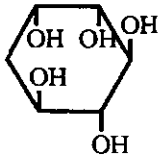
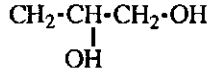
The membrane phospholipids are amphipathic in nature, i.e. they contain a hydrophobic and a hydrophilic region. The basis of the structure of most phospholipids is formed by a glycerol molecule. At the C_1 and C_2 position of the glycerol molecule two fatty acids are esterified. Many phospholipids, occuring in natural membranes, exist as heteroacid lipids, i.e. phospholipids with two different fatty acyl chains, usually a saturated fatty acid at C_1 and an unsaturated fatty acid at C_2. Some phospholipids contain only a fatty acid at the C_1 position and are not esterified at C_2. These are the socalled lysophospholipids. The fatty acyl chain(s) form the hydrophobic region of the molecule.

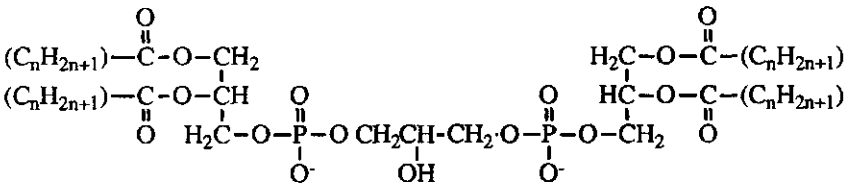
The hydrophilic region is formed by the phosporylalcohol bound to the hydroxyl group at the C_3 position of the glycerol molecule. The common moieties of the phosphoryl alcohols in the endoplasmatic reticulum are phosphorylcholine, phosphorylethanolamine, phosphorylserine and phosphorylinositol. Table B1.1 presents their structural formula. In addition, there are phospholipids which occur less frequent in the endoplasmatic membrane. These phospholipids are

phosphatidylglycerol (PG), containing a phosphorylglycerol at the C_3 position and phosphatidic acid (PA), containing only a phosphate group (PA). In the mitochodria an additional phospholipid, cardiolipin (CL), occurs which is composed of two phosphatidic acid molecules linked together at the phosphate group by a glycerol molecule (Figure B1.1). Sphingomyeline (SM) is the only phospholipid in the membrane that is not derived from glycerol. Instead, the backbone is sphingosine, an amino alcohol that contains a long unsaturated hydrocarbon chain that is part of the hydrophobic domain of the molecule. This phospholipid contains only one fatty acid which is linked to the sphingosine molecule by an amide bond. In addition, the primary hydroxyl group is esterified to phosphocholine and consequently the structure of sphingomyeline resembles that of phosphatidylcholine (Figure B1.1).

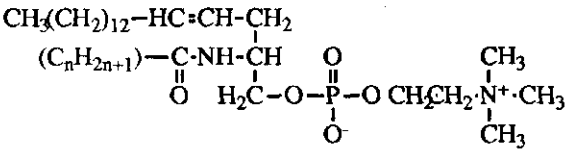
Table B1.1: Chemical structure of glycerol based phospholipids



NAME	STRUCTURE (R)
phosphatidylcholine (PC)	
phosphatidylethanolamine (PE)	
phosphatidylserine (PS)	
phosphatidylinositol (PI)	
phosphatidylglycerol (PG)	
phosphatidic acid (PA)	H



Cardiolipin (CL)



Sphingomyelin (SM)

Figure B1.1: Structure of cardiolipin and sphingomyelin

Because of their amphipathic nature phospholipids tend to orientate in aqueous solutions in a way that keeps the hydrophobic region away from the waterphase. This is usually done by self-assembling into aggregates. The kind of aggregates they form upon solvation in aqueous solutions depends on the molecular shape of the phospholipids. This shape is determined by the relative size of the headgroup compared to the size of the fatty acyl chains and can be either a cone (large headgroup relative to fatty acyl chains such as in for example lysophospholipids), a cilinder (headgroup and fatty acyl chains "equal" in size such as in for example PS, PC and SM) or a wedge or "inverted" cone (small headgroup relative to fatty acyl chains such as in for example unsaturated PE) (Figure B1.2) [11,12]. Cone-shaped lipids show a tendency to organise themselves in micelles, cilinder-shaped lipids in bilayers and wedge-shaped lipids in hexagonal H_{II} phases or reversed micelles (Figure B1.2) [11,12]. Biological membranes are composed of a mixture of cone-, cilinder- and wedge-shaped phospholipids and the overall orientation is that of a bilayer (Figure B1.3).

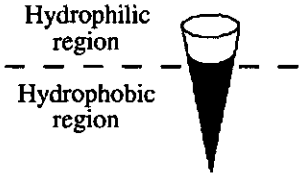
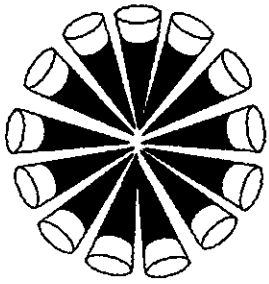
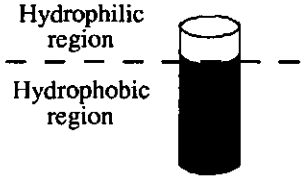
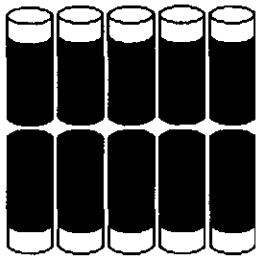
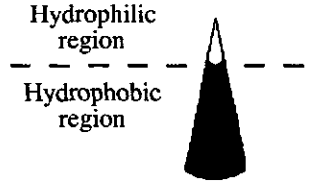
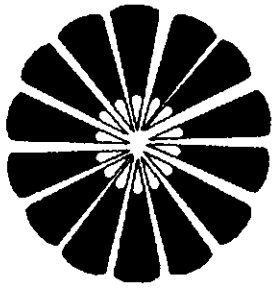
Phospholipid	Shape	Orientation in water
Lyso-phospholipid	 Cone	 Micel
PC, PS, SM and PI	 Cilinder	 Bilayer
PE	 Wedge or "inverted" cone	 Reversed micel

Figure B1.2: Phospholipid shape and orientation in aqueous solutions

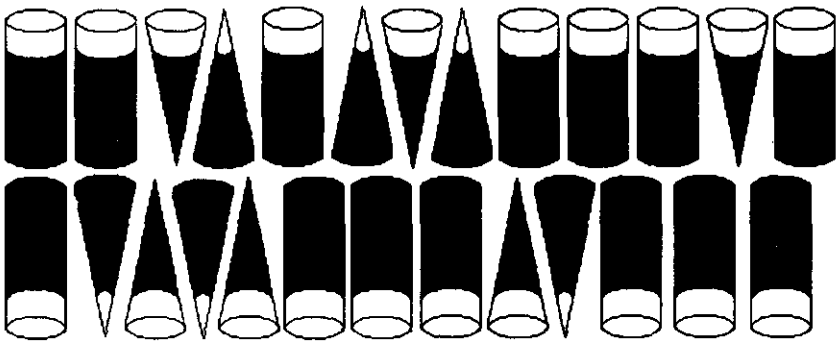


Figure B1.3: Orientation of a mixture of cone-, cilinder- and wedge shaped phospholipids in aqueous solutions

B1.3 Characteristics of the endoplasmatic reticulum membrane

The cytosolic surface of 60% of the endoplasmatic reticulum is dotted with ribosomes. These regions are called the rough endoplasmatic reticulum, in contrast to the regions that contain no attached ribosomes, which are called the smooth endoplamatic reticulum [13,14]. By electronic microscopy the membranes were estimated to be 50-80 Å thick [15,16]. The endoplasmatic reticulum membrane is very dynamic in nature allowing the individual membrane components to be mobile. The mobility is influenced to a substantial degree by the fluidity of the lipid matrix which in turn is mainly determined by the length and degree of unsaturation of the fatty acyl chains of phospholipids, the cholesterol / phospholipid ratio, the amount of PE and SM and the presence of intrinsic membrane proteins [12,17].

About 70% by weight of the endoplasmatic reticulum membrane consists of protein and 30% of lipids. Of these lipids 85% is phospholipid [18] and the remaining 15% is made up of non-phosphorous containing lipids like cholesterol and free fatty acids. The composition of the phospholipid part of the endoplasmic reticulum membrane as reported by Glaumann and Dallner [18] is presented in Table B1.2. The constitution of the phospholipid species of the endoplasmatic reticulum membrane implies that at physiological pH the membrane has an overall negative charge.

The phospholipids of the endoplasmatic reticulum membrane contain a variety of fatty acids. In the endoplasmatic reticulum membrane the fatty acid moiety of phospholipids consists mainly of palmitic acid (from now on referred to as 16:0, i.e. a fatty acid, 16 carbon atoms long containing 0 double (unsaturated) bonds), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), arachidonic acid (20:4) and docosahexaenoic acid (22:6) [19]. The amount of each fatty acid in hepatic microsomal lipids as reported by Borlakoglu *et al.* [20] is presented in Table B1.2.

Table B1.2: Phospholipid and fatty acid composition of the endoplasmatic reticulum membrane

Phospholipid [18]	relative amount (%)	Fatty acid [20]	relative amount (%)
phosphatidylcholine (PC)	55	palmitic acid (16:0)	28
phosphatidyl-ethanolamine (PE)	20-25	palmitoleic acid (16:1)	3
phosphatidylserine (PS)	8-10	stearic acid (18:0)	23
phosphatidylinositol (PI)	5-10	oleic acid (18:1)	15
sphingomyelin (SM)	4-7	linoleic acid (18:2)	13
		arachidonic acid (20:4)	10
		docosahexaenoic acid (22:6)	5
		unidentified	12

A significant proliferation of the endoplasmatic membrane has been reported upon treatment of rats with cytochrome P450 inducers. The total phospholipid concentration, the phospholipid composition and the fatty acid composition have been shown to be affected by phenobarbital, 20-methylcholanthrene and polychlorinated biphenyls [20-23].

Controversy exists with respect to the distribution of the phospholipids over the inner and outer layer of the endoplasmatic reticulum membrane. Some authors reported an asymmetrical distribution with PE and PS predominantly on the outside and PI and SM

predominantly on the inside of the endoplasmatic reticulum membrane [24-26]. Others reported an asymmetrical distribution with PC and SM mainly on the outside and PE and PI mainly on the inside [27-29] whereas Sundler *et al.* [30] reported a symmetrical distribution for all phospholipids.

B1.4 *In vitro* models for the cytochrome P450 enzyme system

The exact structural organisation of the cytochrome P450 enzyme system in the natural endoplasmatic reticulum membrane is presently not well characterised. To allow detailed investigation concerning the effect of phospholipids on the properties of the monooxygenase system, three different types of *in vitro* systems are reported up to now. Before summarizing the effects of phospholipids on the cytochrome P450 system in more detail, each of the three *in vitro* systems will be briefly discussed.

B1.4.1 The microsomal system

Of all three *in vitro* systems, the microsomal system resembles the actual situation in the natural membrane most. Microsomes are small fragments of the endoplasmatic reticulum membrane obtained after extensive homogenisation and differential centrifugation of liver tissue. These fragments seal right side out [24,31-33] to form closed vesicles of approximately 150-300 nm [34]. Thus, the external surface of microsomes corresponds to the cytoplasmatic surface of the endoplasmatic reticulum. Microsomal vesicles mirror the properties of the endoplasmatic reticulum [24,32]. Depending on the part from the endoplasmatic reticulum from which the microsomes originate the vesicles have ribosomes on their outer surface [24]. The composition and distribution of phospholipids and proteins in the vesicle membrane are comparable to those in the endoplasmatic reticulum membrane [24]. Uncharged molecules of low molecular weight (<600-1000 Da) can penetrate microsomal vesicles whereas most charged molecules and high molecular weight proteins do not cross the membrane [35,36]. Compared to the other two *in vitro* systems, the microsomal system is prepared relatively easy and resembles the actual situation in the endoplasmatic reticulum membrane most. However, for mechanistic studies on the

cytochrome P450 system an *in vitro* system is required in which the individual components of the enzyme system can be systematically and easily altered and influenced. This means that the protein components of the enzyme system and phospholipids have to be isolated.

B1.4.2 Reconstituted systems with isolated, membrane incorporated proteins.

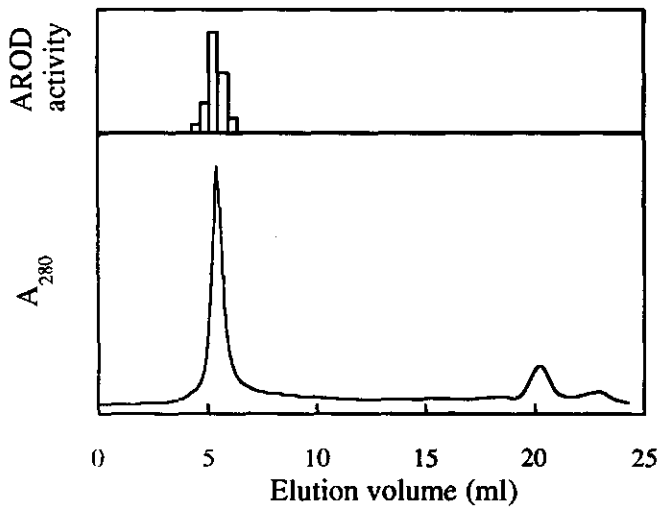


Figure B1.4: FPLC elution pattern of a cholate dialysed preparation of cytochrome P450, NADPH- cytochrome reductase and phospholipids and the alkoxyresorufin-O-dealkylating activity of the fractions (taken from Chapter 2)

This *in vitro* system uses isolated cytochrome P450 and NADPH-cytochrome reductase (and cytochrome b_5 and NADH-cytochrome reductase) which are incorporated into an artificial phospholipid membrane. Unilamellar liposomes with diameters of 50-100 nm are formed upon the removal of cholate from a phospholipid and protein mixture either by dialysis [37] or gel filtration [38]. FPLC experiments demonstrate that the cholate dialysis technique results in the formation of large vesicles of high molecular weight (> 1000 kDa) as can be seen in Figure B1.4. The proteins are inserted in an unidirectional manner with the enzymes orientated toward the outside of the vesicles [39-42]. When

incorporated at a 1:1 stoichiometry cytochrome P450 and NADPH-cytochrome reductase were demonstrated to form heterodimeric complexes of one reductase and one P450 molecule in these vesicles [43,44]. The difference with the microsomal system is that in this *in vitro* system both the protein and the phospholipid composition are well defined and can be changed according to the wishes of the investigator. However, because this *in vitro* system requires isolated proteins it is more difficult to prepare and more artificial than the microsomal system.

B1.4.3. Reconstituted systems with isolated, solubilised proteins

This *in vitro* system also uses the isolated protein components of the cytochrome P450 enzyme system. However, in contrast to the previous (B1.4.2) system, in which the isolated proteins are reincorporated into an artificial membrane, in the present type of *in vitro* system the individual proteins remain water soluble and are mixed with a solution containing phospholipid vesicles. This reconstituted system is obtained by incubating the proteins and phospholipids for a short period (5-10 minutes) in a small volume [45,52]. Generally, this reconstituted system is prepared by incubation of only one form of isolated cytochrome P450, NADPH-cytochrome reductase and phospholipids, although in some cases isolated cytochrome b_5 may also be added. FPLC analysis of the system has shown that the isolated cytochrome P450 and NADPH-cytochrome reductase component both exist as multimeric protein aggregates. Rather than forming heterogenic multimeric protein complexes, cytochrome P450 and NADPH-cytochrome reductase were demonstrated to exist as homomeric protein aggregates of respectively six P450 molecules (IIB1 and IA1) (Figure 2.5) [49,50,52-55] and approximately 31 reductase monomers [50]. For cytochrome P450 LM_2 (IIB1) and LM_4 (IA2) the six monomeric molecules are suggested to be arranged in two layers of three monomers in a 6-pointed star manner [56]. Both protein multimers have a few individual phospholipids attached to each protein multimer [49-51,57].

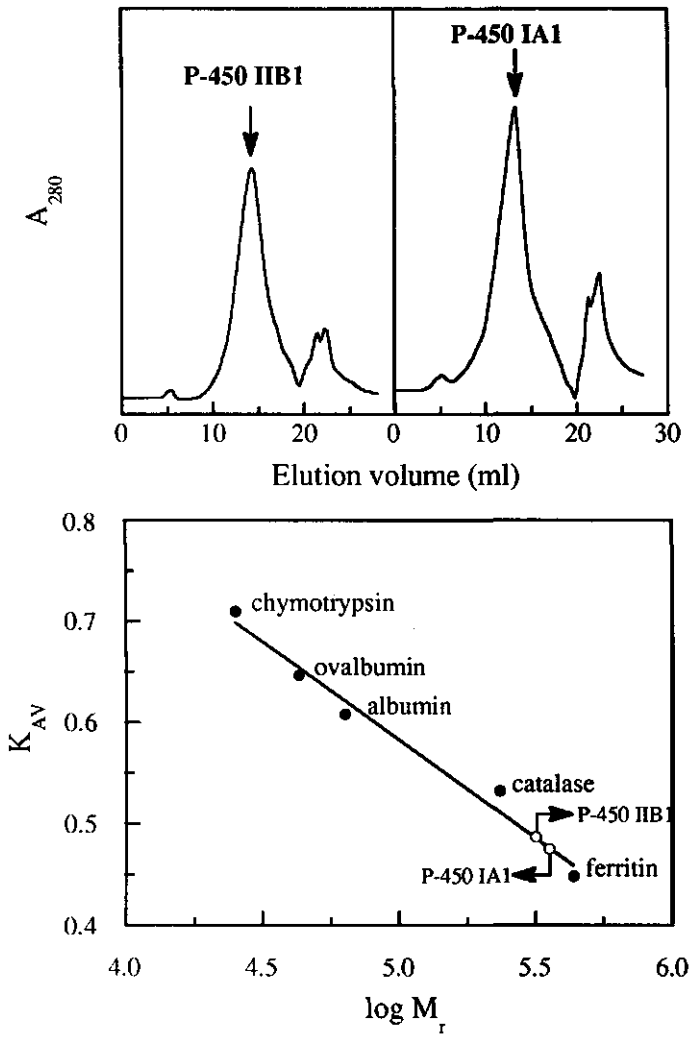


Figure B1.5: FPLC elution pattern of isolated cytochrome P450 IA1 and IIB1 and the calibration curve to determine their aggregation number (taken from Chapter 2)

Early reconstitution experiments in this so-called "solubilised reconstituted in vitro system" [45-47] have demonstrated that cytochrome P450 and NADPH-cytochrome reductase alone are however not sufficient to reconstitute optimal cytochrome P450 dependent conversions. The addition of phospholipids is necessary to obtain optimal cytochrome P450 dependent conversions (see also B1.5). Similar to the reconstituted system in which the proteins were incorporated into a

membrane, in the solubilised reconstituted system the protein and phospholipid compositions can also be changed but because the proteins are not membrane incorporated, this *in vitro* system is the most artificial of all three systems.

B1.5 Phospholipids and cytochrome P450

Since the first succesful reconstitution of cytochrome P450 dependent activities in 1968 [58], the enzyme system has been shown to require at least three endogenous components for optimal reconstitution of cytochrome P450 dependent activities, namely cytochrome P450, NADPH-cytochrome reductase and a microsomal component, stable to heat treatment. This heat stable factor, later identified as phospholipid [45-47] and more specifically phosphatidylcholine [48], was shown to affect the activity of cytochrome P450 dependent reactions, with the reaction rate not only being dependent on the phospholipid concentration but also on the composition of the fatty acyl moiety and the headgroup of the phospholipids [59]. Of several different phosphatidylcholine species tested, the addition of the phosphatidylcholine derivative with two saturated fatty acyl chains of 12 carbon atoms (dilauroyl phosphatidylcholine (PC di12:0)) appeared to result in the highest reconstituted cytochrome P450 dependent activities [59]. Although the stimulating effect of phospholipids on cytochrome P450 dependent activities has been well documented the molecular mechanism underlying this phenomenon is still a matter of considerable debate. A number of hypotheses for the stimulating effect of phospholipids on the cytochrome P450 enzyme system have been proposed which are discussed in more detail hereafter.

B1.5.1 Hypotheses for the stimulating effect of phospholipids on the cytochrome P450 enzyme system

(1) *The membrane provides a lipophilic environment in which the hydrophobic substrates for cytochrome P450 can dissolve*

A low affinity of substrates for cytochrome P450 might in some cases be compensated by partitioning of the substrate into the membrane resulting in a higher effective concentration in the neighbourhood of the active site [2,3]. This assumption was supported by the observation that

incorporation of cytochrome P450 LM_2 (IIB1) from phenobarbital pretreated rabbit liver microsomes into an artificial membrane resulted in a 10-fold decrease in the apparent Michaelis constant (K_m) for benzphetamine [10]. In close relation with this theory it was proposed that the substrate binding site (or the access channel of the substrate binding site) of cytochrome P450 LM_2 from phenobarbital pretreated rabbit liver microsomes was in close contact with the membrane interior [8,9]. Although this hypothesis may very well be of importance for systems in which the proteins of the enzyme system are incorporated into a membrane, it does not explain the stimulating effect of phospholipids in reconstituted systems in which the proteins remain solubilised in an aqueous solution with only a few phospholipid molecules attached to each protein homo-multimer.

(2) *Phospholipids induce a change in the conformation of cytochrome P450*

An effect of phospholipids on the conformation of cytochrome P450 was demonstrated by various investigators using the solubilised reconstituted cytochrome P450 system. Chiang and Coon [60] observed that the presence of phospholipids results in an increase in the helical content of isolated cytochrome P450 LM_2. Furthermore, studies using second derivative spectroscopy demonstrated an increase in the amplitude of the tyrosine band upon interaction of isolated cytochrome P450 LM_2 with phospholipids (and detergents) [61]. The latter observation might best be explained by an increase in the apolar character of the immediate environment of (a) tyrosine residue(s) responsible for the phenomenon. Such an environmental change can be achieved by a conformational change of the protein influencing the tyrosine residue(s) at the surface of the protein. Recently, Rietjens *et al.* [50] observed a decrease in the tryptophan fluorescence polarization of cytochrome P450 IIB1 upon addition of PC di12:0 indicating an increased mobility of this residue in the presence of PC di12:0. This decrease in fluorescence polarization of the single tryptophan residue [62] of P450 IIB1 was suggested to result from a PC di12:0 dependent change in the protein conformation. It should however be mentioned that the addition of PC di18:0 to P450 IIB1 did not result in a significant decrease in the tryptophan fluorescence polarization but this might be related to the much lower ability of this

phospholipid to stimulate cytochrome P450 dependent activities compared to PC di12:0 [50].

(3) *Phospholipids induce a change in the spin-equilibrium of cytochrome P450*

In close relation with the hypothesis described above phospholipids have been reported to cause a shift in the spin equilibrium of cytochrome P450 LM_2 (IIB1) towards the high-spin conformer. Such a shift in the spin equilibrium of cytochrome P450 LM_2 (IIB1) in the presence of PC di12:0 was first observed by Chiang and Coon [60]. In other studies this high-spin shift appeared to be especially favoured by negatively charged phospholipids [63]. A phospholipid induced shift in the spin equilibrium was demonstrated to result in a significant decrease in the apparent binding constant of cytochrome P450 LM_2 for the substrate benzphetamine [64]. Other investigators, however did not observe an altered substrate / enzyme interaction upon the addition of PC di12:0 to cytochrome P450 IIB1 [65-67].

(4) *Phospholipids affect the aggregation state of (artificial) protein multimers*

This model is based on the fact that both isolated cytochrome P450 and NADPH-cytochrome reductase in solution selfaggregate to form homomultimers. Due to its high critical micellar concentration (CMC) of 45 μM [64], PC di12:0 was suggested to act as a detergent and dispers these protein multimers, thereby increasing the effective protein concentration [8,66,67]. However, more recent data demonstrate that it is highly unlikely that the phospholipid induced stimulation of cytochrome P450 dependent activities proceeds according to this mechanism. First, the CMC value of PC di12:0 was demonstrated by Rietjens *et al.* [50] to be two orders of magnitude lower than the one reported by Coon *et al.* [64]. Besides, although high concentrations of Triton X-100, n-octylglucoside or Lubrol PX caused the dissociation of the aggregates [68,69], phospholipids themselves were not able to alter the aggregation state of cytochrome P450 IIB1 significantly [50,70]. Furthermore, the Lubrol PX induced dissociation of the cytochrome P450 IIB1 and NADPH-cytochrome reductase aggregates resulted in a decrease in the pentoxyresorufin-O-dealkylating activity instead of an increase (Figure B1.6) [50]. A new hypothesis by Causey *et al.* [51] suggested that

phospholipids might relax the tight multimeric complexes, thus facilitating functional interactions between P450 and reductase. Results from Figure B1.6, demonstrating an increase in activity at Lubrol concentrations just below concentrations that cause dissociation of the aggregates, seems to support this hypothesis.

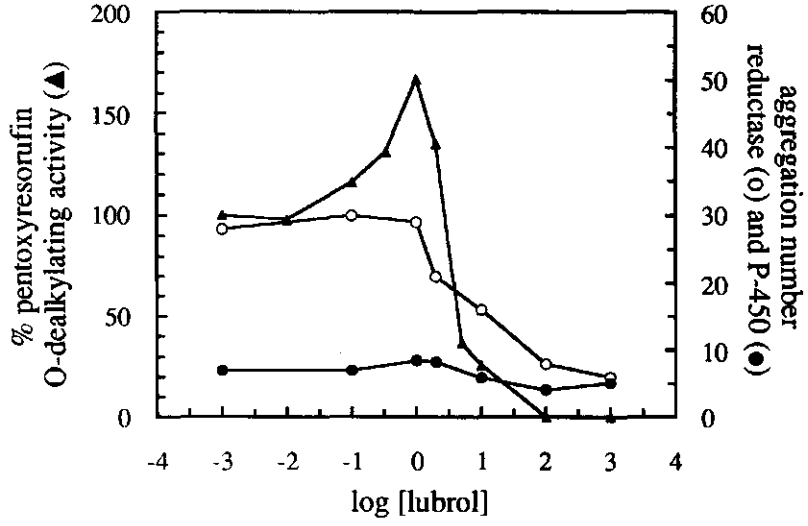


Figure B1.6: The effect of Lubrol PX (concentrations in mM) on cytochrome P450 IIB1 dependent activity and the aggregation state of cytochrome P450 IIB1 and NADPH-cytochrome reductase as determined by FPLC (see fig B1.5). This figure was taken from Rietjens et al. [50]

(5) *Phospholipids facilitate the formation of the catalytically active complex*

The formation of the catalytically active cytochrome P450:NADPH-cytochrome reductase complex can be facilitated by improving the recognition and alignment of the interacting proteins [48]. This can be achieved through hydrophobic interactions between proteins and the membrane phospholipids or through neutralization of repelling charges on the surface of cytochrome P450 and NADPH-cytochrome reductase by electrostatic interactions between charged amino acid residues on the protein surfaces and phospholipid headgroup. The dilauroyl derivative of phosphatidylcholine (PC di12:0) has been demonstrated to facilitate complex formation by decreasing the apparent dissociation constants of the cytochrome P450 / reductase complex [64,67,71]. Especially the

negatively charged phospholipids appeared to favour the formation of the cytochrome P450 / reductase electron exchange complex [7].

B1.5.2 Specific cytochrome P450 - phospholipid interactions

Because of the importance of phospholipids for the cytochrome P450 enzyme system the existence of specific lipid-protein interactions in the cytochrome P450 system has been a topic for investigation in several studies. For the mitochondrial cytochrome P-450_{scc} a specific requirement has been reported for cardiolipin [72]. It was demonstrated that this mitochondrial cytochrome P450 form contained a specific effector site for cardiolipin [73]. For microsomal cytochrome P450 the existence of specific lipid-protein interactions is still a subject of controversy. Early reports suggested that the in rabbit liver microsomes the cytochrome P450 / NADPH-cytochrome reductase complex is enclosed by a halo of phospholipids which differs from the bulk phospholipid matrix [74]. More recently, specific interactions have been suggested between cytochrome P450 LM_2 (IIB1) and a number of phospholipid species such as phosphatidic acid (PA) [4] and phosphatidylethanolamine (PE) [75,76]. The interactions with the negatively charged PA have been suggested to be based on the charge-charge coupling of the negative charge on the phospholipid headgroup and positive charges on the protein surface. The interactions with PE on the other hand, have been suggested to originate from the wedge-shaped PE structure which might adapt cytochrome P450 specifically to the membrane [77]. Specific interactions with PS, which is well known for its ability to interact with positive charges on protein surfaces, have not yet been reported for cytochromes P450 although the stimulating effect of PS on the catalytic activity of reconstituted cytochrome P450 [5,78] points to a possible relationship between cytochromes P450 and PS.

B1.6 Scope of this thesis

In the paragraph above a number of hypotheses for the stimulating effect of phospholipids on the cytochrome P450 enzyme system have been described. However, despite the amount of work done on this topic over the last 25 years the exact molecular mechanism of phospholipid stimulation of vertebrate cytochrome P450 dependent activities is still not understood. This is at least to some extent due to the complexity of the

enzyme system and the diversity in effects induced by phospholipids. Important questions like:

(i) what structural aspects of membrane phospholipids affect cytochrome P450 dependent substrate conversions,

(ii) how do phospholipids affect different forms of cytochrome P450,

(iii) do phospholipids affect the other protein constituents of the enzyme system as well and

(iv) do the cytochrome P450 forms exhibit specific affinities for certain phospholipids

still remain to be answered.

The scope of this thesis was to gain further insight into the role(s) of the membrane and (membrane)phospholipids in the cytochrome P450 enzyme system. This thesis is however, not solely focussed on the cytochrome P450 protein but also pays attention to NADPH-cytochrome reductase.

Chapters 2 to 7 describe the results of the experiments as they were executed. The first chapters (Chapters 2, 3 and 4) deal with the relation between cytochrome P450 and phospholipids. Chapter 5 discusses the relation between NADPH-cytochrome reductase and phospholipids and Chapters 6 and 7 describe a side reaction that can occur during cytochrome P450 dependent substrate conversion; NADPH-cytochrome reductase catalysed redox cycling. Finally, in Chapter 8 of this thesis the results are summarized and evaluated and conclusions are presented.

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Chapter 2

Differential cumene hydroperoxide sensitivity of cytochrome P450 enzymes IA1 and IIB1 determined by their way of membrane incorporation

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2.1 Summary

The cytochrome P450-dependent O-dealkylation of alkoxyresorufins was used to study the effect of cumene hydroperoxide on cytochrome P450 IIB1 and IA1 in microsomal and reconstituted systems.

In liver microsomal systems from respectively phenobarbital and 3-methylcholanthrene pretreated male Wistar rats, cytochrome P450 IIB1-dependent pentoxyresorufin O-dealkylation appeared to be more sensitive to cumene hydroperoxide treatment than cytochrome P450 IA1-dependent ethoxyresorufin O-dealkylation. This phenomenon was also observed when the cumene hydroperoxide sensitivity of P450 IIB1 and IA1 was studied in an isosafrole pretreated rat liver microsomal system. The decrease in alkoxyresorufin O-dealkylating activities appeared to proceed by destruction of the cytochrome P450 component of the enzyme system.

Purification and reconstitution of the enzyme system components in a system in which the isolated proteins were not incorporated into a membrane resulted in the disappearance of the difference in sensitivity between the two P450 enzymes . However, in a reconstituted system with membrane incorporated proteins, again cytochrome P450 IIB1 expressed a higher sensitivity towards cumene hydroperoxide than cytochrome P450 IA1.

From this it was concluded that the differential cumene hydroperoxide sensitivity of cytochrome P450 IIB1 and IA1 is not caused by an intrinsic difference in their sensitivity but by a differential effect of membrane incorporation on their cumene hydroperoxide sensitivity.

2.2 Introduction

The membrane bound cytochrome P450 system, located in the endoplasmatic reticulum of mainly the liver, is involved in the monooxygenation of a broad range of endogenous substrates and xenobiotics. It is made up of several components, namely cytochrome P450, NADPH-cytochrome reductase, NADH-cytochrome reductase, cytochrome b_5 and phospholipids. During biotransformation reactions, the cytochrome P450 system can produce reactive oxygen species, that may inactivate the cytochromes P450 and are considered to be initiators

of lipidperoxidation [1,2]. The lipid hydroperoxides, resulting from this process have also been reported to inactivate cytochrome P450 dependent substrate conversions by oxidizing the heme group of the protein [3-7]. Furthermore, inactivation of microsomal cytochromes P450 has been observed under other conditions of "oxidative stress" like exposure to ozone [8], hydrogen peroxide [9] and organic hydroperoxides like cumene and tert.butyl hydroperoxide [10]. With these organic hydroperoxides the oxidative stress may result from radicals formed during the homolytic cleavage of the peroxides at the active site of the cytochromes P450 [11-14].

Studies demonstrating the cytochrome P450 sensitivity to conditions of oxidative stress generally do not focus on a possible different sensitivity of the various cytochrome P450 enzymes. Such a difference, however, can be important from a toxicological point of view. Especially when one considers the possible bioactivation of a compound by one P450 enzyme and its conversion to a non-toxic metabolite by another [15]. In the literature, only a few studies point to a possible differential sensitivity of cytochromes P450 towards conditions of oxidative stress. Gibson and Schenkman [16] and Jefferey *et al.* [17] mentioned the possible existence of two populations of cytochrome P450 that differed in their susceptibility to destruction by linoleic acid hydroperoxide, but these populations were not further characterized. In a study using lung microsomes an increased sensitivity of the lung cytochrome P450(s) catalyzing pentoxyresorufin O-dealkylation as compared to the P450 enzyme(s) catalyzing the O-dealkylation of ethoxyresorufin was demonstrated [8]. Recently, Kitada *et al.* [18] provided evidence that Sprague-Dawley rat liver microsomal cytochrome P450 PB-1 is more susceptible to lipidperoxidation than cytochromes P450d (IA2) and P450h (IIC11). Furthermore, in a study from Sesardic *et al.* [19] it was shown that in male Wistar rats, cytochrome P450d (IA2) was more sensitive to treatment with CCl_4 than cytochrome P450c (IA1).

The present study was undertaken to investigate possible differences in sensitivity between cytochromes P450 IIB1 and IA1 towards cumene hydroperoxide. Cytochrome P450 enzymes IIB1 and IA1 were used because they could be studied in microsomal form as well as in isolated form in reconstituted systems.

In this paper it is demonstrated that microsomal cytochrome P450 IIB1 is more sensitive towards cumene hydroperoxide than microsomal

cytochrome P450 IA1. Possible factors underlying this different cumene hydroperoxide sensitivity were investigated, using the isolated P450 enzymes in two types of reconstituted systems.

2.3 Materials and methods

2.3.1 Preparation of microsomes

Microsomes were prepared from the perfused livers of male Wistar rats (ca. 300 g), pretreated with phenobarbital (Brocacef b.v., Maarssen, The Netherlands) (0.1% in drinking water for 7 days), 3-methylcholanthrene (Sigma, St. Louis, MO, USA) (30 mg/kg bodyweight, using a stock solution of 6 mg/ml in olive oil, i.p., daily for 3 days) and isosafrole (Janssen, Beerse, Belgium)(150 mg/kg bodyweight, using a stock solution of 100 mg/ml in olive oil, i.p., daily for 3 days). Following homogenization of the livers in Tris/sucrose buffer (50 mM Tris, 0.25 M sucrose pH 7.4) and centrifugation at 10,000 x g (20 min), the supernatants were centrifuged for 75 minutes at 105,000 x g. The microsomal pellet was washed once with Tris/sucrose buffer and finally resuspended in 0.1 M potassium phosphate pH 7.25, containing 20% glycerol and 0.1 mM EDTA, immediately frozen into liquid nitrogen and stored at -90 °C until use.

2.3.2 Biochemical assays

The concentration of cytochrome P450 was determined as described by Omura and Sato [20]. Cytochrome b_5 concentrations were measured using the method of Omura and Sato [21]. NADPH- and NADH-cytochrome reductase activities were determined as described by Phillips and Langdon [22], using cytochrome *c* (Boehringer, Mannheim, FRG) as the electron acceptor. Protein concentrations were measured using the method of Lowry *et al.* [23] using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard. Lipidperoxidation was measured by the thiobarbituric acid assay, described by Buege and Aust [24]. Lipid extraction from liver microsomes from phenobarbital pretreated rats was carried out according to the method of Bligh and Dyer [25]. The extracted lipids were stored under argon at -20 °C until use.

2.3.3 Purification of microsomal enzymes

NADPH-cytochrome P450 reductase was isolated from liver microsomes of phenobarbital pretreated female Wistar rats essentially as described by Yasukochi and Masters [26], using the same minor modifications as described by Rietjens *et al.* [27]. Excess detergent and phospholipid was removed by binding the protein to the 2'5'ADP-sepharose column equilibrated with 20 mM potassium phosphate, pH 7.7, containing 20 % glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol and 0.1 mM phenylmethanesulfonyl fluoride (Merck, Darmstadt, FRG) followed by washing with at least 10 column volumes of equilibration buffer and elution of the protein with the same buffer, containing 10 mM adenosine 2'(3')-monophosphate (Sigma, St. Louis, MO, USA) and 0.2% sodium deoxycholate (Merck, Darmstadt, FRG). The reductase preparation was concentrated over an Amicon YM-30 filter and dialyzed against 3 times 2 litres of 20 mM potassium phosphate pH 7.7, 20% glycerol, 0.1 mM EDTA and 0.1 mM dithiothreitol to remove the deoxycholate and adenosine 2'(3')-monophosphate. The final detergent-free preparation had a specific activity of 36.7 units/mg protein and was homogeneous as judged by SDS-PAGE, carried out as described by Laemmli [28].

Cytochrome P450 IIB1 was isolated from liver microsomes of phenobarbital pretreated male Wistar rats essentially as described by West *et al.* [29] using Lubrol PX (Sigma, St. Louis, MO, USA) instead of Emulgen 911. Excess detergent was removed by additional washing of the hydroxylapatite column bound protein using the prescribed buffers without detergents. The final detergent-free preparations contained 8-12 nmoles of P450 heme/mg protein.

Cytochrome P450 IA1 was isolated from liver microsomes of 3-methylcholanthrene pretreated male Wistar rats, essentially as described by Ryan *et al.* [30] using Lubrol PX instead of Emulgen 911. Excess detergent was removed as described above for cytochrome P450 IIB1. The final detergent-free preparations contained 8-12 nmoles of P450 heme/mg protein. Both cytochrome P450 preparations were over 95% pure as judged by SDS-PAGE and were identified as cytochrome P450 IA1 and IIB1 in an immunoblotting experiment using the monoclonal antibodies described by Letawe-Goujon *et al.* [31] (not shown).

The purified, detergent-free cytochrome P450 preparations were further characterized by FPLC gel filtration as described below.

2.3.4 FPLC gel filtration

FPLC gel filtration was carried out using a Superose 6 column (18 x 1 cm) (Pharmacia, Uppsala, Sweden) in 0.1 M potassium phosphate pH 7.5, at a flow rate of 0.5 ml/min with detection at 280 nm.

2.3.5 Microsomal and reconstituted cytochrome P450 for *in vitro* incubations

Microsomes were diluted before use in 0.1 M potassium phosphate pH 7.25 containing 20% glycerol and 0.1 mM EDTA. In the microsomal assay 50 µl of the diluted microsomes, containing 0.125 nmol cytochrome P450, was used.

The reconstituted system with solubilized enzymes was prepared essentially as described before [27]. In short, 0.125 nmol cytochrome P450, 0.31 units NADPH-cytochrome reductase and 20 µg of distearoyl phosphatidylcholine were preincubated in a small volume (50 µl) for 10 minutes at 37 °C before buffers, substrate and NADPH were added up to a volume of 1.0 ml.

The reconstituted system with enzymes incorporated into an artificial membrane was prepared by cholate dialysis essentially as described by Taniguchi *et al.* [32]. In short, a solution, containing 2.5 nmol/ml cytochrome P450, 6.3 units/ml NADPH-cytochrome reductase and 0.31 mg/ml microsomal phospholipid, prepared from liver microsomes of phenobarbital pretreated rats as described above, in 50 mM potassium phosphate pH 7.25, 0.1 mM EDTA, 0.1 mM dithiothreitol and 1% (w/v) sodium cholate was dialyzed for 2x24 hr against 2x2 liters of the same buffer without cholate. In the assay 50 µl of the dialyzed solution, containing 0.125 nmol P450, was used.

2.3.6 Exposure of microsomal and reconstituted cytochrome P450 systems to cumene hydroperoxide

In the microsomal systems, 10 µl of a cumene hydroperoxide (Aldrich, Steinheim, FRG) solution were added to 50 µl of diluted microsomal solution, to give the final hydroperoxide concentration, depicted in the figures and tables. Cumene hydroperoxide was diluted in methanol. After 10 minutes of incubation at 37 °C, pentoxy- and ethoxyresorufin O-dealkylating activities were measured using the method of Burke *et al.* [33]. In short, 0.1 M (final concentration) potassium phosphate pH 7.5, 5 mM (final concentration) $MgCl_2$ and 5 µM (final concentration) of the

alkoxyresorufin (10 µl of a 0.5 mM stock solution in Me_2SO/ml assay) were added. The reaction was started by addition of NADPH (0.4 mM final concentration).

In the reconstituted system with solubilized enzymes, 10 µl of a cumene hydroperoxide solution were added to the 50 µl preincubation mixture. After 10 minutes buffers and substrate were added and the reaction was started by addition of NADPH, all as described above.

In the reconstituted system with membrane-incorporated enzymes, 50 µl of the dialyzed solution were incubated for 10 minutes at 37 °C with 10 µl of a hydroperoxide solution After this incubation the other reagents were added as described above.

Pentoxy- and ethoxyresorufin O-dealkylating activities are expressed as % of control unless indicated otherwise. Control activities being the activity measured after 10 minutes preincubation with 10 µl methanol.

2.3.7 Statistical analysis of data

Data are presented as the mean ± standard error of the mean (SEM) and statistical analysis was carried out using Student's t-test, for unpaired samples. The null-hypothesis was rejected at $P \leq 0.05$.

2.4 Results

2.4.1 Pentoxy- and ethoxyresorufin O-dealkylating activities in different microsomal systems

In Figure 2.1 the pentoxy- and ethoxyresorufin O-dealkylating activities in liver microsmes from phenobarbital pretreated, 3-methylcholanthrene pretreated, isosafrole pretreated and untreated male Wistar rats (control) are presented. These data demonstrate that a respective 4- and 30-fold increase in the pentoxyresorufin O-dealkylating activity (PROD) was obtained after treatment with respectively isosafrole and phenobarbital compared to control

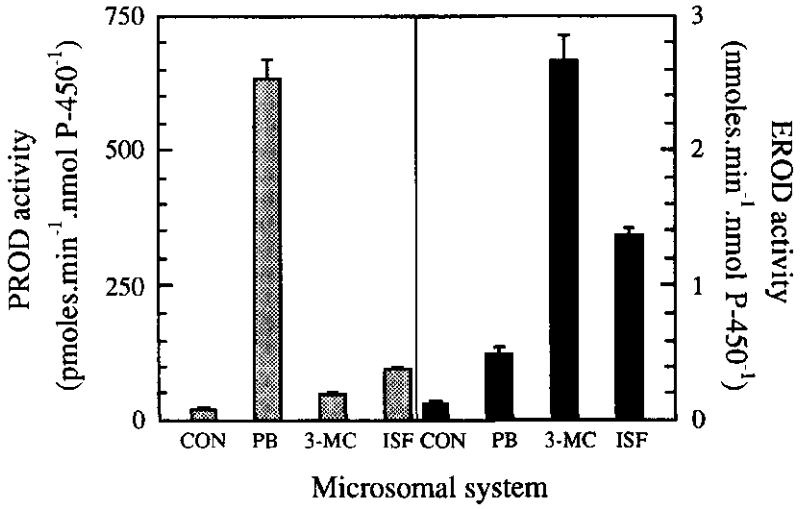

Figure 2.1: Pentoxy- and ethoxyresorufin O-dealkylating activity in liver microsomal systems from phenobarbital, 3-methylcholanthrene and isosafrole pretreated rats and untreated male Wistar rats.

Pentoxy- and ethoxyresorufin O-dealkylating activities were determined as described in 'Materials and methods'. The results represent the mean ± SEM of two different microsomal preparations each tested in triplicate.

CON = liver microsomes of untreated rats; PB = liver microsomes of phenobarbital pretreated rats; 3-MC = liver microsomes of 3-methylcholanthrene pretreated rats ; ISF = liver microsomes of isosafrole pretreated rats.

microsomes. Furthermore, a 11- and 20-fold increase in the ethoxyresorufin O-dealkylating activity (EROD) was obtained after treatment with respectively isosafrole and 3-methylcholanthrene. This effect of the inducers on the pentoxy- and ethoxyresorufin O-dealkylating activity in microsomal systems has been described before by several authors [33-35]. From these results, together with the fact that (i) phenobarbital and 3-methylcholanthrene greatly increase the level of respectively P450 IIB1 and IA1 in rat liver microsomes [36] and (ii) antibodies against P450 families IIB and IA have been shown to decrease the pentoxy- and ethoxyresorufin O-dealkylation in respectively phenobarbital and 3-methylcholanthrene pretreated rat liver microsomes

by respectively >90% and 79% [37,38] , it was concluded that in liver microsomes from phenobarbital and 3-methylcholanthrene pretreated rats 80-90% of the respective O-dealkylating activities can be ascribed to IIB1 and IA1. Based on similar arguments it was concluded that in liver microsomes of isosafrole pretreated rats the major part (>50%) of the respective activities can be ascribed to IIB1 and IA1 activity.

2.4.2 Conditions for cumene hydroperoxide-dependent oxidative stress

Figure 2.2 shows the time dependent inactivation of pentoxy- and ethoxyresorufin O-dealkylation in liver microsomes from isosafrole pretreated rats exposed to 0.9 mM (final concentration) cumene hydroperoxide.

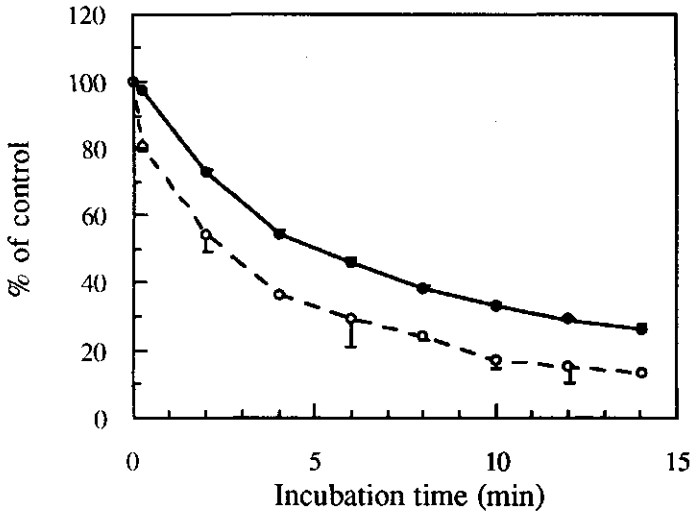

Figure 2.2: Time dependent inactivation of pentoxy- and ethoxyresorufin O-dealkylating activity in a liver microsomal system from isosafrole pretreated male Wistar rats.

0.125 nmol of microsomal cytochrome P450 was incubated at 37 °C for increasing time intervals in a small volume (60 µl) with 0.9 mM CuOOH (final concentration). The pentoxy- and ethoxyresorufin O-dealkylation were determined as described in 'Materials and methods'. (o-o) = pentoxyresorufin O-dealkylation. (●-●) = ethoxyresorufin O-dealkylation. The results represent the mean ± SEM of four experiments. Based on this experiment an incubation time of 10 minutes was chosen for further experiments.

2.4.3 Cumene hydroperoxide sensitivity of cytochromes P450 IIB1 and IA1 in microsomal systems

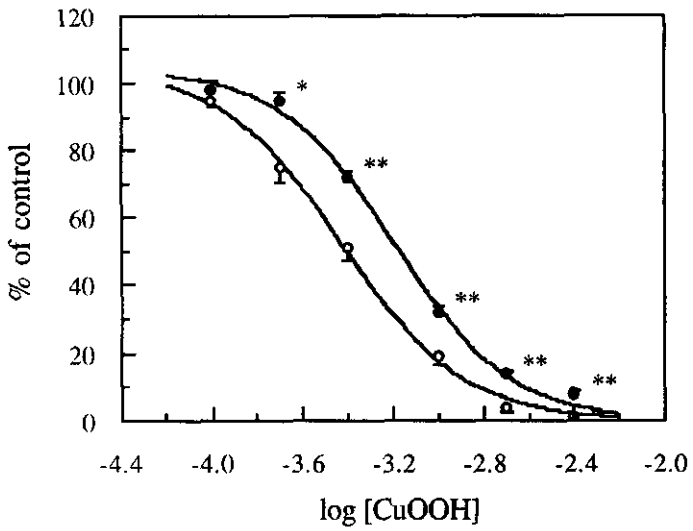

Figure 2.3: CuOOH-sensitivity of alkoxyresorufin O-dealkylating activities in a microsomal system from isosafrole pretreated male Wistar rats.

0.125 nmol of microsomal cytochrome P450 was incubated for 10 minutes at 37 °C with varying concentrations of cumene hydroperoxide (in M) in a small volume (60 µl) before activities were determined. Pentoxy- and ethoxyresorufin O-dealkylating activities were determined as described in 'Materials and methods'. (o-o) = pentoxyresorufin O-dealkylation. (●-●) = ethoxyresorufin O-dealkylation. The results represent the mean ± SEM of two independent microsomal preparations each tested in quadruplicate.

* significantly different from the value for the pentoxyresorufin O-dealkylation at $P<0.02$

** significantly different from the value for the pentoxyresorufin O-dealkylation at $P<0.001$

Figure 2.3 demonstrates the cumene hydroperoxide dependent inactivation of alkoxyresorufin O-dealkylations in liver microsomes from isosafrole pretreated rats. From these curves EC-50 values, defined as the cumene hydroperoxide concentrations causing 50% inactivation, can be determined. Table 2.1 summarizes EC-50 values obtained from experiments in which the cumene hydroperoxide sensitivity of pentoxy-

and ethoxyresorufin O-dealkylation in three different microsomal systems was studied.

Table 2.1 *EC-50 values for the cumene hydroperoxide dependent inactivation of microsomal pentoxy- and ethoxyresorufin O-dealkylation.*

inducer	EC-50 (mM)	
	pentoxyresorufin O-dealkylation	ethoxyresorufin O-dealkylation
phenobarbital	0.11 ± 0.01	n.d.
3-methylcholanthrene	n.d.	0.27 ± 0.01*
isosafrole	0.41 ± 0.03	0.67 ± 0.03**

Liver microsomes from rats pretreated with different inducers were used. n.d. = not determined. Values presented are the mean ± SEM of two independent preparations.
* significantly different from the EC-50 value for the pentoxyresorufin O-dealkylating activity in phenobarbital pretreated rat liver microsomes at $P < 0.001$.
** significantly different from the EC-50 for the pentoxyresorufin O-dealkylating activity in this microsomal system at $P<0.001$.

From the data, presented in Figure 2.3 and Table 2.1, it appears that microsomal cytochrome P450 IIB1 is significantly more sensitive towards inactivation by cumene hydroperoxide than cytochrome P450 IA1. In theory, the difference in cumene hydroperoxide sensitivity between pentoxyresorufin O-dealkylation in phenobarbital pretreated rat liver microsomes and the ethoxyresorufin O-dealkylation in 3-methylcholanthrene pretreated rat liver microsomes might be caused by differences in lipid composition, P450 enzyme composition or antioxidant content between the two microsomal systems. Results from the isosafrole pretreated microsomal system, however, excluded this possibility.

Additional experiments demonstrated that inactivation of the cytochrome P450 dependent activities was accompanied by a simultaneous formation of thiobarbituric acid reactive materials (Figure 2.4), indicating the occurence of lipidperoxidation in these microsomes. Furthermore, a decrease in the microsomal cytochrome P450 content was shown to be responsible for the decrease in the alkoxyresorufin O-dealkylating activity (Figure 2.4). The other components of the enzyme system, cytochrome b_5, NADPH- and NADH-cytochrome reductase,

were not inactivated at these cumene hydroperoxide concentrations (data not shown).

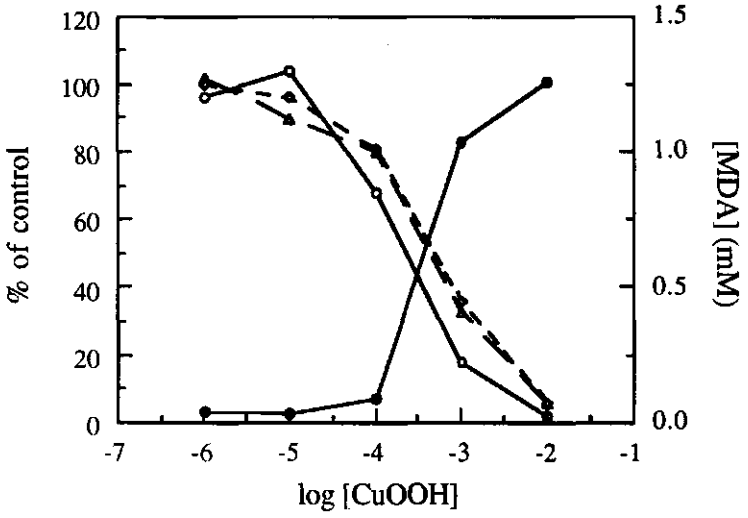

Figure 2.4: Influence of cumene hydroperoxide on the cytochrome P450 levels and the amount of TBA reactive material in microsomes from phenobarbital, 3-methylcholanthrene and isosafrole pretreated male Wistar rats.

0.125 nmol of microsomal cytochrome P450 was incubated for 10 minutes at 37 °C with varying concentrations of cumene hydroperoxide before the cytochrome P450 content and the amount of malondialdehyde was determined. The amount of MDA (●-●) at varying hydroperoxide concentrations was comparable in all three microsomal systems and the results depicted in this figure represent the average of the three microsomal systems. The P450 level is expressed as % of control. Control level being the amount of P450 measured after 10 minutes incubation with 10 µl methanol. (o-o) cytochrome P450 content in PB microsomes, (Δ-Δ) cytochrome P450 content in 3-MC microsomes, (◊-◊) cytochrome P450 content in ISF microsomes.

2.4.4 Characteristics of isolated cytochromes P450 IIB1 and IA1

To test whether the differential cumene hydroperoxide sensitivity of the cytochromes P450 IIB1 and IA1, was due to intrinsic differences in their sensitivities, the effect of the hydroperoxide on isolated cytochromes P450 was investigated.

First, the isolated P450 preparations were characterized with respect to their purity, aggregation state and the effects of detergents on

their cumene hydroperoxide sensitivity to determine whether they were qualitatively comparable. The cytochrome P450 preparations contained 8-12 nmoles of P450 heme per mg protein and were over 95% pure as judged by SDS-PAGE. Figure 2.5[a] shows the FPLC elution pattern of the purified cytochrome P450 preparations on a Superose 6 column. From these elution patterns and the calibration curve of the Superose 6 column given in Figure 2.5[b] it can be calculated that, in solubilized form, both cytochromes form aggregates. Cytochrome P450 IIB1, identified by pentoxyresorufin O-dealkylating activity in a reconstituted system, appears to elute in one peak (elution volume 14.10 ± 0.06 ml) with a mean apparent molecular mass of 317 ± 32 kDa. Using the value of 52 kDa for its monomeric form [29], the detergent free cytochrome P450 IIB1 turns out to exist as an aggregate of six polypeptide chains. This is in agreement with results presented earlier [27,39,40].

The elution pattern of cytochrome P450 IA1 also shows one peak (elution volume 13.89 ± 0.06 ml), containing ethoxyresorufin O-dealkylating activity in a reconstituted system and thus identified as cytochrome P450 IA1. The apparent molecular mass of this peak was determined to be 358 ± 28 kDa. Using 56 kDa for the monomeric form of cytochrome P450 IA1 [30], this indicates aggregates of also six polypeptide chains, which has not yet been reported before in the literature for cytochrome P450 IA1 from rat liver.

In addition, the FPLC data demonstrate the protein preparations to have similar purities. The nature of the low molecular weight impurities ($M_r = \pm 3$ kDa), present in both preparations, is not further characterized.

As the presence of detergent after purification might, in theory, influence the sensitivity of the P450 enzymes to cumene hydroperoxide, the effect of artificially added detergents on the hydroperoxide sensitivity of the P450 enzymes was also studied. For both preparations it was shown that the presence of neither lubrol nor cholate in concentrations up to at least 10% of their concentration in the isolation buffers did influence the cumene hydroperoxide sensitivity of the cytochrome P450 enzymes (data not shown). This implies that residual detergent in the protein preparations, expected to be far less than 10% of the concentration in the buffers of the isolation procedure, will not affect the EC-50 values for the cumene hydroperoxide sensitivity.

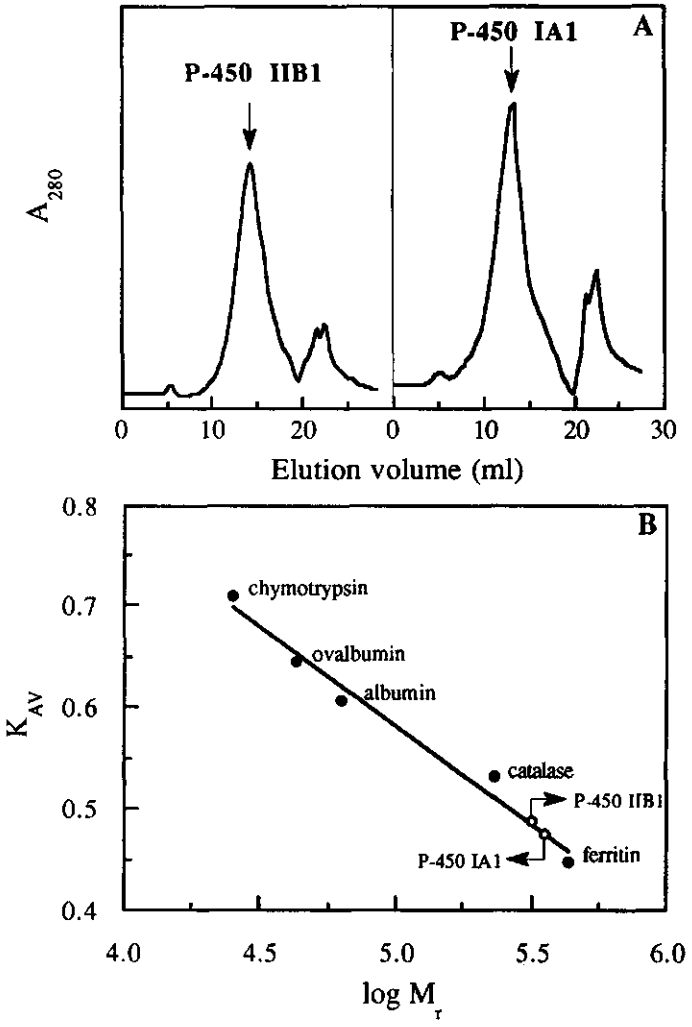

Figure 2.5: Determination of the apparent molecular mass of cytochrome P450 IIB1 and IA1 using FPLC gel filtration on Superose 6 in 0.1 M potassium phosphate pH 7.5

(A) Elution pattern of cytochrome P450 IIB1(100 µl of a solution containing 2 nmol cytochrome P450 / ml) and cytochrome P450 IA1 (100 µl of a solution containing 2 nmol cytochrome P450 / ml). The peaks containing O-dealkylating activity in a reconstituted system are indicated by arows. (B) Calibration cutve of the Superose 6 column using proteins of known molecular mass and the determination of the molecular mass of isolated cytochrome P450 IIB1 and IA1. K_{AV} is a distribution coefficient and defined as: $K_{AV} = (V_e - V_0) / (V_t - V_0)$ with V_e = elution volume, V_0 = void volume (6.13 ml) and V_t = total column volume (22.58 ml).

Alltogether the data, described above, demonstrate that both cytochrome P450 preparations are comparable with respect to their purity, specific heme content, aggregation state and the absence of an effect of residual detergents on their cumene hydroperoxide sensitivity.

2.4.5 Cumene hydroperoxide sensitivity of cytochrome P450 IIB1 and IA1 in reconstituted systems

Table 2.2: EC 50 values for cumene hydroperoxide dependent inactivation of isolated cytochromes P450 IIB1 and IA1 in reconstituted systems in which the proteins are not membrane bound ("solubilized") and in reconstituted systems in which the proteins are incorporated into an artificial membrane by cholate dialysis ("membrane incorporated").

	EC-50 (mM)	
	pentoxyresorufin O-dealkylation (P450 IIB1)	ethoxyresorufin O-dealkylation (P450 IA1)
solubilized reconstituted system	0.20 ± 0.03	0.19 ± 0.02 n.s.
membrane bound reconstituted system	0.09 ± 0.01 *	0.30 ± 0.01 *** **

Values given are the mean ± SEM of 2 independent preparations each measured in quadruplicate. n.s. = not significant

* significantly different from the value for the solubilized reconstituted system at P<0.005.

** significantly different from the value for the solubilized reconstituted system at P<0.001.

*** significantly different from the value for the pentoxyresorufin O-dealkylation at P<0.001

Table 2.2 lists EC 50 values for cumene hydroperoxide dependent inactivation of the cytochrome P450 dependent activities in the two reconstituted systems. From the data presented it can be concluded that in a reconstituted system in which the isolated enzymes are not incorporated into a membrane, there is no difference in their cumene hydroperoxide sensitivity. The absence of a difference in cumene hydroperoxide sensitivity in this reconstituted system did not result from the use of

distearoyl phosphatidylcholine in the reconstitution; similar results were obtained when phospholipids, extracted from microsomes were used (data not shown). In this reconstituted system, as in the microsomal system, destruction of cytochrome P450 was responsible for the decrease in the alkoxyresorufin O-dealkylating activity; NADPH-cytochrome reductase activity being not affected (data not shown). The reconstituted system, used in the present study, contains reductase:cytochrome P450 ratio's that are about 15 times higher (1:1) than the ratio reported for microsomes (about 1:15) [41]. The 1:1 ratio for cytochrome P450 and NADPH-cytochrome reductase in reconstituted systems has been used before [40,42]. Further experiments demonstrated that decreasing this reductase / P450 ratio from 1:1 to 1:10, in order to make it more comparable to the ratio in the microsomal system, did not affect the EC 50 values (data not shown).

In the second reconstituted system, the cholate dialysis technique was used to incorporate the proteins into an artificial membrane, made up of phospholipids, extracted from liver microsomes from phenobarbital pretreated rats. Figure 2.6 presents the FPLC elution pattern of one of these preparations. Cytochrome P450 dependent activity eluted as one, large peak in the void volume of the column, indicating an apparent molecular mass higher than 10,000 kDa. In addition, the elution pattern in figure 2.6 demonstrated a small peak, containing no O-dealkylating activity, with an apparent molecular mass of about 4 kDa, probably resulting from protein free lipid clusters. From the FPLC elution pattern of figure 2.6 it can be concluded that both the cytochrome P450 and the NADPH-cytochrome reductase are incorporated into the large phospholipid vesicle. Furthermore, the membrane incorporated enzyme preparations were free from cytochrome P-420, the inactive form of cytochrome P450.

From the EC-50 values presented in Table 2.2 it can be derived that as a result of membrane incorporation of cytochrome P450 IIB1 and IA1 the difference in their cumene hydroperoxide sensitivity reappears ($P<0.001$). Upon membrane incorporation, cytochrome P450 IIB1 becomes 2.1 times more sensitive ($P<0.001$) towards cumene hydroperoxide than in the solubilized reconstituted system. On the other hand, cytochrome P450 IA1 becomes 1.6 times less sensitive ($P<0.005$) than in the solubilized reconstituted system. Finally, from Table 2.2 it appears that the EC-50 values for the ethoxy- and pentoxyresorufin O-

dealkylation by membrane incorporated, isolated cytochrome P450 IA1 and IIB1 are almost identical to the EC-50 values for the respective activities in the 3-methylcholanthrene and phenobarbital microsomal system from which they were isolated (Table 2.1).

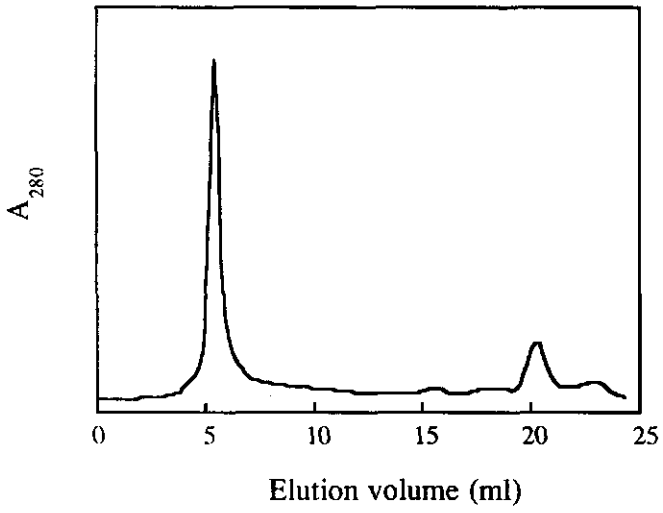

Figure 2.6: FPLC gel filtration pattern of membrane incorporated, reconstituted cytochrome P450.

Elution pattern of isolated cytochrome P450 IIB1 and NADPH-cytochrome reductase, reconstituted in an artificial membrane of extracted endoplasmatic reticulum phospholipids (50 µl of a solution containing 0.9 nmol cytochrome P450/ml and 2.25 units reductase/ml) on Superose 6 in 0.1 M potassium phosphate pH 7.5

2.5 Discussion

In the present study microsomal cytochrome P450 IIB1-dependent pentoxyresorufin O-dealkylation and cytochrome P450 IA1-dependent ethoxyresorufin O-dealkylation were used to study the possible differential sensitivity of cytochromes P450 towards cumene hydroperoxide. The difference in sensitivity between the two P450 enzymes towards this hydroperoxide was demonstrated in microsomal systems. For these systems it is important to stress that pentoxy- and ethoxyresorufin are not completely specific substrates for these P450 enzymes. Nevertheless, based on literature data [33-38] it was concluded

that in liver microsomes from phenobarbital and 3-methylcholanthrene pretreated rats over 80-90% of the respective activities can be ascribed to IIB1 and IA1, whereas in liver microsomes of isosafrole pretreated rats the major part (>50%) of the respective activities can be ascribed to IIB1 and IA1 activity.

The results from the experiments on the difference in sensitivity between P450 IIB1 and IA1 in microsomal systems demonstrated cytochrome P450 IIB1 to be more sensitive towards cumene hydroperoxide treatment than cytochrome P450 IA1. Comparing the EC-50 values in the microsomal systems it appears that in the isosafrole microsomal system the difference in hydroperoxide sensitivity is smaller than the difference observed, when comparing the phenobarbital and 3-methylcholanthrene microsomal systems. This phenomenon is most likely related to the lower induction of cytochrome P450 IIB1 and IA1 in isosafrole microsomes [36]. Due to this lower induction larger parts of the O-dealkylating activities have to be ascribed to P450 enzymes other than P450 IIB1 and IA1. This could partly mask their difference in hydroperoxide sensitivity.

In theory, the different sensitivities of cytochromes P450 IIB1 and IA1 can be caused by differences in intrinsic characteristics between the P450 enzymes, such as for example their affinity for cumene hydroperoxide, their capacity for homolytic cleavage of this peroxide, or their sensitivity to the reactive intermediates. On the other hand the difference in sensitivity can also be caused by external factors such as their way of membrane incorporation, membrane surroundings and antioxidant surroundings, determining the exposure and accessibility of their active sites.

To investigate these possibilities to a further extent the present study describes results from experiments in which the peroxide sensitivity of isolated cytochromes P450 IIB1 and IA1 was determined in two different reconstituted systems. Determination of the cumene hydroperoxide sensitivities of cytochromes P450 IIB1 and IA1 in reconstituted systems in which the proteins were not incorporated into an artificial membrane, demonstrated that the cytochrome P450 enzymes were equally sensitive, thereby excluding an intrinsic difference in peroxide sensitivity between the two P450 enzymes as a possible explanation for the observed difference in microsomal systems. Incorporation of the isolated P450 enzymes into an artificial membrane

resulted in a considerable effect on their sensitivity towards cumene hydroperoxide. Cytochrome P450 IIB1 became significantly ($P<0.001$) more sensitive compared to solubilized reconstituted P450 IIB1, whereas cytochrome P450 IA1became significantly ($P<0.005$) less sensitive than solubilized reconstituted P450 IA1. It is interesting to note that the EC-50 values for the O-dealkylating activities in this reconstituted system are nearly identical to those obtained for the phenobarbital and 3-methylcholanthrene microsomal systems, from which the respective P450 enzymes were isolated. This indicates that the isolation procedure did not affect the cumene hydroperoxide sensitivities of the P450 enzymes.

From these results it was concluded that the mechanism underlying the difference in cumene hydroperoxide sensitivity between P450 IIB1 and IA1, as observed in the microsomal and membrane incorporated reconstituted system was a differential effect of membrane incorporation on their structure and/or the accessibility of their active sites. A difference in the way of membrane association between rabbit liver microsomal cytochrome P450 LM_3 (IIIA6) and LM_4 (IA2) has been reported by Ingelman-Sundberg and Glaumann [43]. Their conclusion was based on the observation that reconstituted cytochrome P450 LM_3 formed vesicles with larger diameters than P450 LM_4. Furthermore, both solubilized and membrane bound P450 LM_4 could be denatured by the non-penetrating reagent diazobenzene sulphonate, whereas membrane bound P450 LM_3 was not susceptible to the diazo-reagent.

Over the last years the membrane topology of cytochromes P450 has been a topic for many researchers. Contrary to earlier predictions, in which the active site and the majority of the polypeptide chain was believed to be deeply embedded in the microsomal membrane [44-46], recent data favor an orientation with only one or two membrane spanning helices at the N-terminus and the main part of the protein located in the cytoplasm [47-50]. Substrates would gain access to the active site directly from the membrane in which they are believed to be concentrated, as a result of their hydrophobic nature. A different effect of membrane incorporation on the cumene hydroperoxide sensitivity of cytochrome P450 IIB1 and IA1, as demonstrated in this study, could result from a different orientation and thus accessibility of their active sites with respect to the membrane. One could imagine cumene hydroperoxide gaining access to the active site of cytochrome P450 IIB1 through the membrane whereas it gains access to the active site of cytochrome P450

IA1 through the water phase. Cumene hydroperoxide, being hydrophobic, tends to partition into the apolar core of the membrane thereby increasing the effective concentration in the vicinity of the active site of cytochrome P450 IIB1, resulting in the higher sensitivity of this P450 enzyme, when incorporated into a membrane. Taking the proteins out of the membrane would result in equal accessibilities and therefore equal sensitivities of P450 IIB1 and IA1.

However, since there is no experimental evidence to support the model described above, other explanations for the observed difference in hydroperoxide sensitivity between membrane bound cytochromes P450 IIB1 and IA1 have to be considered. The effects observed might also be explained by a different effect of membrane incorporation on the protein structures, thereby changing their intrinsic sensitivities in a different way, or by a different lipid environment for the two P450 enzymes. Further studies will be necessary to discriminate between these explanations. Nevertheless, the results presented clearly demonstrate that membrane incorporation can influence two cytochrome P450 enzymes in a different way.

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Chapter 3

Kinetics of cytochromes P450 IA1 and IIB1 in reconstituted systems with dilauroyl- and distearoyl-glycerophosphocholine

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3.1 Summary

In the present study the effect of changing the fatty acyl moiety of phosphatidylcholine from dilauroyl to distearoyl on the kinetic parameters of O-dealkylation of alkoxyresorufins and ethoxycoumarin dependent on reconstituted cytochrome P450 IA1 and IIB1 has been investigated. The results demonstrate that i) the maximum rate of O-dealkylation (V_{max}) for both P450 enzymes was about two times higher in the L-α-dilauroyl-*sn*-glycero-3-phosphocholine (PC di12:0) system compared with the L-α-distearoyl-*sn*-glycero-3-phosphocholine (PC di18:0) system and ii) changes in the fatty acyl moiety of phosphatidylcholine (PC) from dilauroyl to distearoyl affected the apparent K_m for the substrate (K_m^s) of P450 IA1 and IIB1 in a different way. In addition, (iii) the kinetic parameters appeared to be dependent on the PC : P450 ratio and a change in this ratio affected the kinetic parameters of P450 IA1 and IIB1 in a different manner. From these last two observations it was concluded that the mechanism by which phospholipids influence P450 IA1 dependent O-dealkylation of ethoxycoumarin is different from that by which they influence P450 IIB1 dependent O-dealkylation of this substrate. Furthermore, the results of the present study demonstrate that the increase in the rate of O-dealkylation of ethoxycoumarin, reported in the literature for reconstituted systems in the presence of PC di12:0, results from an effect of PC di12:0 on both the K_m^s and the V_{max}.

In a number of additional experiments possible mechanisms underlying the observed differential effect of PC di12:0 and PC di18:0 on the K_m^s and V_{max} of P450 IA1 and IIB1 were investigated. This was done by studying the effect of the two PC species on the kinetic parameters of some of the different steps of the P450 cycle, namely substrate binding, oxygen binding and the rate of electron transfer. The results demonstrate an influence of PC di12:0 and PC di18:0 on 1) substrate binding to cytochrome P450, 2) the affinity of cytochromes P450 for NADPH-cytochrome reductase and thus on 3) the electron flow through the reconstituted system. Based on the results from these experiments it was concluded that the increased V_{max} of P450 IA1 and IIB1 in the presence of PC di12:0 compared to the systems with PC di18:0 was at least in part due to an increased affinity of both P450 enzymes for NADPH-cytochrome reductase in

the presence of PC di12:0 compared to PC di18:0. With respect to the differential effect of PC di12:0 and PC di18:0 on the K_m^s of P450 IA1 and IIB1 the results demonstrate that this phenomenon does not result from a differential effect of the two PC species on either the substrate binding, the oxygen binding or the rate of electron transfer. Therefore, it was concluded that the differential effect of PC di12:0 and PC di18:0 on the K_m^s of P450 IA1 and IIB1 must result from an effect of the PC species on the kinetic parameters of other steps in the P450 cycle such as reductive oxygen splitting, substrate conversion and / or product release.

3.2 Introduction

The cytochrome P450 enzyme system is involved in the monooxygenation of a broad range of endogenous substrates and xenobiotics. Associated to the membrane of the endoplasmatic reticulum, the properties of this enzyme system, such as the rates and specificities of the reactions it catalyzes, may be influenced by the nature of the membrane. Especially membrane fluidity (determining the lateral and rotational mobilities of the proteins), membrane charge (determining the interaction between ionic groups on the proteins) and the hydrophobic character of the core of the membrane (contributing to substrate accumulation in the vicinity of the enzymes) have been demonstrated to influence the cytochrome P450 enzyme system [1-9]. These physical properties of the membrane are determined by the membrane phospholipids; membrane charge is dependent on the headgroups of the phospholipids and fluidity and hydrophobicity are influenced by the fatty acid substituents.

Already in the late 1960's, it was demonstrated that reconstitution of cytochrome P450 dependent activities required, besides cytochrome P450 and NADPH-cytochrome reductase, a so called 'heat stable factor' [10,11], later identified as phosphatidylcholine [12,13]. Subsequent to the identification of phosphatidylcholine as the active lipid factor, different types of phospholipid were investigated for their stimulatory properties on P450 dependent reactions. The phosphatidylcholine with acyl = dilauroyl was found to be most efficient [14,15] and thereafter it was

chosen by many investigators for studies with reconstituted P450 systems.

In theory, the differences observed in substrate conversion rates in the presence of various phosphatidylcholine species might be a reflection of both changes in K_m^s for the xenobiotic substrate as well as in V_{max} characteristics of the reconstituted cytochrome P450 systems. For the cytochrome P450 IIB1 conversion of pentoxyresorufin, for instance, apparent V_{max} and K_m^s values for the substrate were different in the presence of PC di12:0 or PC di18:0 [16]. The stimulating effect of PC di12:0 on the V_{max} for P450 dependent substrate conversions was studied in a number of investigations and could be caused by an increased efficiency of electron transfer from NADPH-cytochrome reductase (E.C.1.6.2.4) to cytochrome P450. This increased electron transfer may result from i) changed protein conformations, ii) a decreased apparent binding constant for the catalytically active reductase : cytochrome P450 complex (K_d^r) and / or iii) changes in the spin state of cytochrome P450 in the presence of PC di12:0 [16-20].

In contrast, the effect of different phosphatidylcholine species on the apparent K_m for the substrate (K_m^s) has not been studied in detail. This in spite of the fact that in theory an effect of phosphatidylcholine species on the affinity of the P450 for the substrate i.e. the binding constant for the P450 : substrate complex (K_d^s), resulting from a role of the phospholipids in the binding of substrates to the active site of the cytochrome P450 molecule, might be reflected in an influence on the K_m^s. In addition, an effect of phosphatidylcholine species on other kinetic parameters of the P450 cycle might also result in an influence on the K_m^s. An effect of the phosphatidylcholine species on the K_m^s and / or K_d^s value could be of considerable importance, especially because in living organisms substrate concentrations will generally be low. In addition, in systems containing multiple P450 enzymes, a different effect of PC species on the substrate apparent K_m^s of different P450 enzymes, converting a substrate to different metabolites, may not only influence the rate of metabolism but also the metabolite pattern observed.

The objective of the present study was to investigate the effect of a change in the fatty acyl moiety of phosphatidylcholine on the kinetic parameters (especially K_m^s and K_d^s) for substrate conversion

by two cytochrome P450 enzymes. For these studies isolated reconstituted cytochromes P450 IA1 and IIB1 were used as the model P450 enzymes, PC di12:0 and PC di18:0 as the model phosphatidylcholine species and ethoxyresorufin, pentoxyresorufin and ethoxycoumarin as the substrates.

3.3 Materials and methods

3.3.1 Preparation of microsomes

Microsomes were prepared from the perfused livers of Wistar rats (ca. 300 g) which were pretreated with phenobarbital (Brocacef b.v., Maarssen, The Netherlands) (0.1% in drinking water) or ß-naphthoflavone (Sigma, St. Louis, MO, USA) (30 mg/kg body mass, using a stock solution of 12.5 mg/ml in olive oil, intraperitoneally, daily for 3 days). Following homogenization of the livers in Tris/sucrose buffer (50 mM Tris , 0.25 M sucrose pH 7.4) and centrifugation at 10,000 x g (20 min), the supernatants were centrifuged for 75 minutes at 105,000 x g. The microsomal pellet was suspended in 0.1 M potassium phosphate pH 7.25, containing 20% glycerol and 0.1 mM EDTA, immediately frozen into liquid nitrogen and stored at -90 °C until use for isolation of NADPH-cytochrome reductase, cytochromes P450 IA1 or IIB1.

3.3.2 Purification and characterization of NADPH-cytochrome reductase and cytochromes P450 IA1 and IIB1

NADPH-cytochrome P450 reductase was isolated from liver microsomes of phenobarbital pretreated female Wistar rats essentially as described by Yasukochi and Masters [21], using the same minor modifications as described before [16], and using Emulgen 911 (KAO Chemicals, Tokyo, Japan) instead of Lubrol PX. Excess detergent and phospholipid was removed by binding the protein to a column of adenosine 2'5'-bisphosphate linked to sepharoses, equilibrated with 20 mM potassium phosphate, pH 7.7, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol (Boehringer, Mannheim, FRG) and 0.1 mM phenylmethanesulfonyl fluoride (Merck, Darmstadt, FRG) followed by washing with at least 10 column volumes of equilibration buffer and elution of the protein with the same buffer containing 10

mM adenosine 2'(3')-monophosphate (Sigma, St.Louis, MO, USA) and 0.2% sodium deoxycholate (Merck, Darmstadt, FRG). The reductase preparation was concentrated over an Amicon YM-30 filter and dialyzed three times against 2 l of 20 mM potassium phosphate pH 7.7, 20% glycerol, 0.1 mM EDTA and 0.1 mM dithiothreitol to remove the deoxycholate and adenosine 2'(3')-monophosphate. The final detergent-free preparation had a specific activity of 37.8 U/mg protein and was homogeneous as judged by SDS/PAGE, carried out as described by Laemmli [22]. One unit (U) of NADPH-cytochrome reductase activity is the amount required to catalyze the reduction of 1 μmol cytochrome *c* / min.

Cytochrome P450 IA1 was isolated from liver microsomes of b-naphthoflavone pretreated male Wistar rats essentially as described by Ryan *et al.* [23], using Lubrol PX (Sigma, St.Louis, MO, USA) instead of Emulgen 911. Excess detergent was removed by additional washing of the hydroxy apatite column bound protein, using the prescribed buffers without detergents. The final detergent-free preparation contained 9 nmol P450 heme/mg protein.

Cytochrome P450 IIB1 was isolated from liver microsomes of phenobarbital pretreated rats, essentially as described by West *et al.* [24] using Lubrol PX instead of Emulgen 911. Excess detergent was removed as described above for cytochrome P450 IA1. The final detergent-free preparation contained 14 nmol P450 heme/ mg protein. Both cytochrome P450 preparations were over 90% pure as judged by SDS/PAGE. The purified cytochrome P450 preparations were identified as cytochrome P450 IA1 and IIB1 in an immunoblotting experiment using the monoclonal antibodies described by Letawe-Goujon *et al.* [25].

3.3.3 Biochemical assays

The concentration of cytochrome P450 was determined from the CO-difference spectrum of the dithionite-reduced protein, based on an absorption coefficient of 91 $mM^{-1}.cm^{-1}$ as described by Omura and Sato [26]. NADPH-cytochrome reductase activities were determined as described by Phillips and Langdon [27], using cytochrome *c* (Boehringer, Mannheim, FRG) as the final electron acceptor. Protein concentrations were measured using the method of Lowry *et al.* [28]

using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard.

Pentoxy- and ethoxyresorufin O-dealkylating activities were measured using the method of Burke *et al.* [29] in 0.1 M potassium phosphate pH 7.5 at 37 °C using 0.1-5 μM (as indicated) of the alkoxyresorufins (Boehringer, Mannheim, FRG) added in dimethylsulfoxide (Me_2SO,10 μl /ml assay). The activities were quantified upon calibration of the fluorescence scale by the addition of resorufin (10 μl of a 104 μM solution in Me_2SO), the product of the cytochrome P450 dependent O-dealkylation of alkoxyresorufins.

Ethoxycoumarin O-dealkylating activities were measured essentially as described by Ullrich and Weber [30] in 0.1 M Tris/HCl pH 7.6 at 37 °C using 10-100 μM (as indicated) of ethoxycoumarin (Janssen Chimica, Beerse, Belgium) added in Me_2SO (10 μl/ml assay). The activities were quantified upon calibration of the fluorescence scale by the addition of hydroxycoumarin (10 μl of a 103 μM solution in Me_2SO), the product of the cytochrome P450 dependent O-dealkylation of ethoxycoumarin.

The O-dealkylating activities were determined on an SLM/Aminco SPF-500c spectrofluorometer and are expressed as rate of resorufin or hydroxycoumarin formation / molar amount of cytochrome $P450^{-1}$.

3.3.4 Reconstituted incubations

The reconstituted system for the ethoxy- and pentoxyresorufin-O-dealkylation was prepared as described before [16]. In short, 0.01 nmol cytochrome P450, 0.025 U NADPH-cytochrome reductase and 2 μg of either PC di12:0 or PC di18:0 (both from Sigma, St. Louis, MO, USA) were preincubated in a small volume (50 μl) for 6 minutes at 37 °C before buffer, substrate and NADPH (0.4 mM final concentration) were added up to a volume of 1 ml. The O-dealkylating activity was measured as described above.

The reconstituted system for the ethoxycoumarin O-dealkylation was essentially the same as the reconstituted system for the alkoxyresorufin O-dealkylation, only 10 times higher concentrations of the P450 system components were used. In short, 0.1 nmol of cytochrome P450, 0.25 U of reductase and 20 μg of phosphatidylcholine (unless indicated otherwise) were preincubated in

a small volume (50 µl) for 6 minutes at 37 °C before buffer, substrate and NADPH (0.4 mM final concentration) were added up to a volume of 1 ml. The O-dealkylating activity was measured as described above.

3.3.5 Determination of the influence of phospholipids on the apparent binding constant of cytochrome P450 IIB1 and IA1 for ethoxycoumarin (K_d^s)

In order to obtain conditions similar to those in the kinetic experiments the apparent K_d values of cytochrome P450 IA1 and IIB1 for ethoxycoumarin (K_d^s) were determined in the presence of NADPH-cytochrome reductase. The K_d^s values were obtained from the substrate induced shift in the spin equilibrium of the cytochrome P450. For determination of the K_d^s of cytochrome P450 for ethoxycoumarin, 1 µM P450, 1 µM reductase and 320 µM PC di12:0 or 240 µM PC di18:0 were preincubated for 15 min. at 25 °C in a small volume (200 µl). After preincubation 0.1 M potassium phosphate buffer (pH 7.5) containing 20 % glycerol was added to the incubation mixture up to a volume of 1 ml. The K_d^s was determined by the stepwise addition of a 10 µM solution of ethoxycoumarin in 0.1 M Tris/HCl pH 7.5 upto 15 µl. After each addition the absorption spectrum between 350 and 450 nm was recorded on an SLM/Aminco DW-2000 spectrophotometer at 25 °C against a blank cuvet filled with buffer to which similar stepwise additions of ethoxycoumarin were made. The spectra were corrected for the absorption spectrum of P450 and reductase without added ethoxycoumarin. The substrate apparent binding constants were calculated from the double reciprocal plots of $(\text{ethoxycoumarin concentration})^{-1}$ against $(\Delta A_{390-420})^{-1}$.

3.3.6 Statistical analysis of data

Data are presented as the mean ± standard error of the mean and statistical analysis was carried out using unpaired Students' t-test. The null-hypothesis was rejected at $P \leq 0.05$.

3.4 Results

3.4.1 The influence of PC di12:0 and PC di18:0 on the kinetic parameters of cytochrome P450 dependent O-dealkylation of alkoxyresorufins and ethoxycoumarin

In Table 3.1 the K_m^s and V_{max} of the ethoxy- and pentoxyresorufin-O-dealkylation by cytochrome P450 IA1 and IIB1 in the PC di12:0 and PC di18:0 reconstituted systems are presented.

Table 3.1: Apparent substrate K_m^s and V_{max} of ethoxy- and pentoxyresorufin O-dealkylation by cytochromes P450 IA1 and IIB1 in the presence of either PC di12:0 or PC di18:0.

reconstituted cytochrome P450	assay	phospholipid	K_m^s (μM)	V_{max} (nmol. min^{-1}. nmol $P450^{-1}$)
IA1	EROD	PC di12:0	0.36 ± 0.03	13.8 ± 1.6
		PC di18:0	0.63 ± 0.05***	8.0 ± 0.8*
IIB1	PROD	PC di12:0	4.8 ± 0.4	0.38 ± 0.06
		PC di18:0	2.3 ± 0.2***	0.23 ± 0.02**

The O-dealkylation of pentoxyresorufin (PROD) and ethoxyresorufin (EROD) by reconstituted cytochromes P450 IIB1 and IA1 was determined as described in 'Materials and methods'. The P450 / reductase / PC molar ratio in the reconstituted system was 1 : 1: 320 in the PC di12:0 system and 1 : 1 : 240 PC di18:0 system. The results presented are the mean ± standard error of the mean of six to nine experiments with two independent P450 preparations.

* significantly different from the value with PC di12:0 at P<0.005

** significantly different from the value with PC di12:0 at P<0.002

*** significantly different from the value with PC di12:0 at P<0.001

From these data it can be seen that for both P450 enzymes the V_{max} in the presence of PC di12:0 is 1.7 times higher than in the presence of PC di18:0. However, cytochrome P450 IA1 and IIB1 exhibit an opposite behaviour with respect to the effect of PC di12:0 and PC di18:0 on the K_m^s for the alkoxyresorufins. Reconstitution of cytochrome P450 IA1 with PC di12:0 decreases the K_m^s for ethoxyresorufin by a factor of almost two compared to the PC di18:0 system. On the other hand, reconstitution of cytochrome P450 IIB1

with PC di12:0 results in a twofold increase of the K_m^s for pentoxyresorufin compared to the value observed in the PC di18:0 system.

Table 3.2: K_m^s and V_{max} of ethoxycoumarin-O-dealkylation by cytochromes P450 IA1 and IIB1 in the presence of either PC di12:0 or PC di18:0.

reconstituted cytochrome P450	assay	phospholipid	K_m^s (μM)	V_{max} (nmol. min^{-1}. nmol $P450^{-1}$)
IA1	ECOD	PC di12:0	28 ± 3	9.2 ± 0.8
		PC di18:0	45 ± 4*	5.6 ± 0.2**
IIB1	ECOD	PC di12:0	201 ± 22	3.3 ± 0.3
		PC di18:0	92 ± 11***	2.1 ± 0.2*

The O-dealkylation of ethoxycoumarin (ECOD) by reconstituted cytochrome P450 IIB1 and IA1 was determined as described in 'Materials and methods'. The P450 / reductase / PC molar ratio in the reconstituted system was 1:1:320 in the PC di12:0 system and 1:1:240 PC di18:0 system. The results presented are the mean ± standard error of the mean of eight experiments with two independent P450 preparations.

* significantly different from the value for PC di12:0 at P<0.01

** significantly different from the value for PC di12:0 at P<0.005

*** significantly different from the value for PC di12:0 at P<0.001

In theory this differential effect of the phospholipids on the K_m^s for ethoxy- and pentoxyresorufin of cytochrome P450 IA1 and IIB1 might be caused by the different structures of the substrates. To determine whether this was the mechanism underlying the observed differential effect on the K_m^s, the experiments were repeated with ethoxycoumarin as a substrate for both P450 IA1 and IIB1. The kinetic parameters obtained from these experiments are summarized in Table 3.2. From these data it can be seen that the effects observed for the ethoxy- and pentoxyresorufin O-dealkylation are also observed for the ethoxycoumarin O-dealkylation. Both P450 enzymes exhibit a 1.6 times higher V_{max} in the presence of PC di12:0 compared to the PC di18:0 system. In addition, for cytochrome P450 IA1 PC di12:0 causes a decrease in the substrate K_m^s compared to PC di18:0 whereas

for cytochrome P450 IIB1 PC di12:0 increases the K_m^s compared to PC di18:0. The values for the K_m^s of P450 IA1 and IIB1 for ethoxycoumarin in the PC di12:0 system are almost identical to those obtained by Miwa and Lu [31] for the ethoxycoumarin-O-dealkylation by these P450 enzyme systems, incorporated in an artificial membrane of egg yolk phosphatidylcholine.

3.4.2 Influence of the phosphatidylcholine / cytochrome P450 ratio on the kinetics of the O-dealkylation of ethoxycoumarin

So far, the results presented describe kinetic experiments using cytochrome P450, NADPH-cytochrome reductase and phospholipid in a molar ratio of 1:1:240 for the PC di18:0 system and 1:1:320 for the PC di12:0 system. The effects of varying the phospholipid : P450 ratio on the K_m^s and V_{max} of the P450 dependent O-dealkylation of ethoxycoumarin were also determined. The kinetic data obtained from these experiments are presented in figures 3.1 and 3.2.

Figure 3.1[a] clearly demonstrates that for cytochrome P450 IA1 an increase in the PC : P450 ratio from 0 to about 25 for both phospholipids is accompanied by a significant decrease in the K_m^s for ethoxycoumarin. Further increasing the PC : P450 ratio did not further affect the K_m^s. From Figure 3.1[b] it can be seen that increasing the ratio also decreases V_{max} although in the case of PC di12:0 the decrease is relatively small.

The effect of an increasing PC : P450 ratio on the K_m^s and V_{max} of the P450 IIB1 dependent O-dealkylation of ethoxycoumarin appeared to be significantly different .

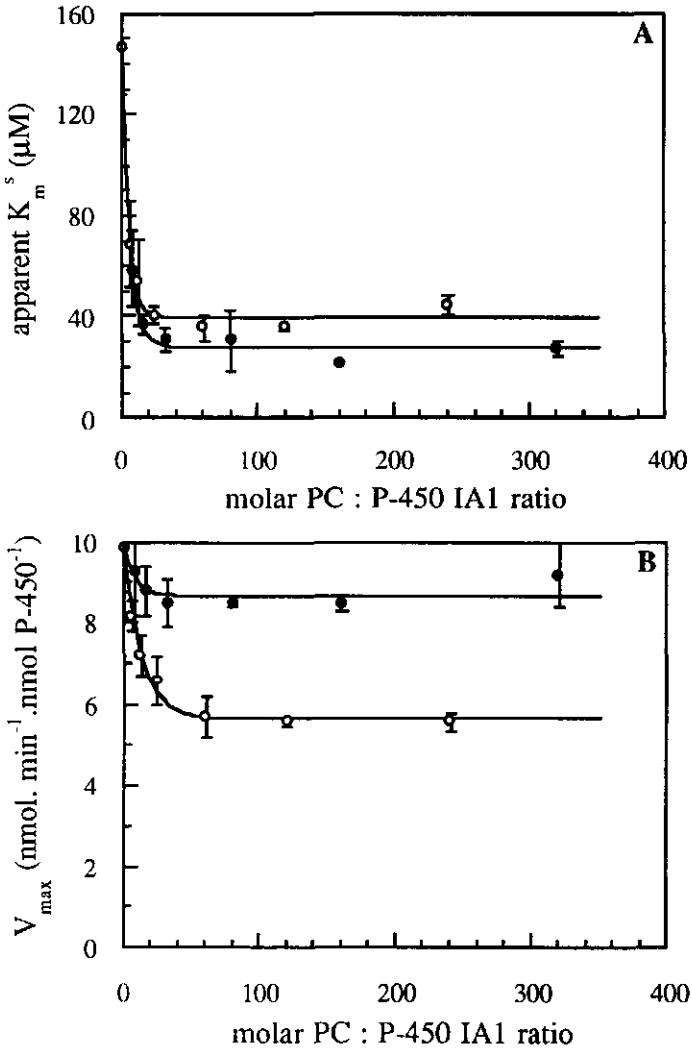

Figure 3.1: The effect of changes in the PC / P450 ratio on the K_m^S (a) and V_{max} (b) of cytochrome P450 IA1 for ethoxycoumarin in the PC di12:0 (●-●) and PC di18:0 (o-o) systems.

Ethoxycoumarin-O-dealkylating activities were measured as described in 'Materials and Methods'. The results represent the mean ± standard error of the mean of two experiments.

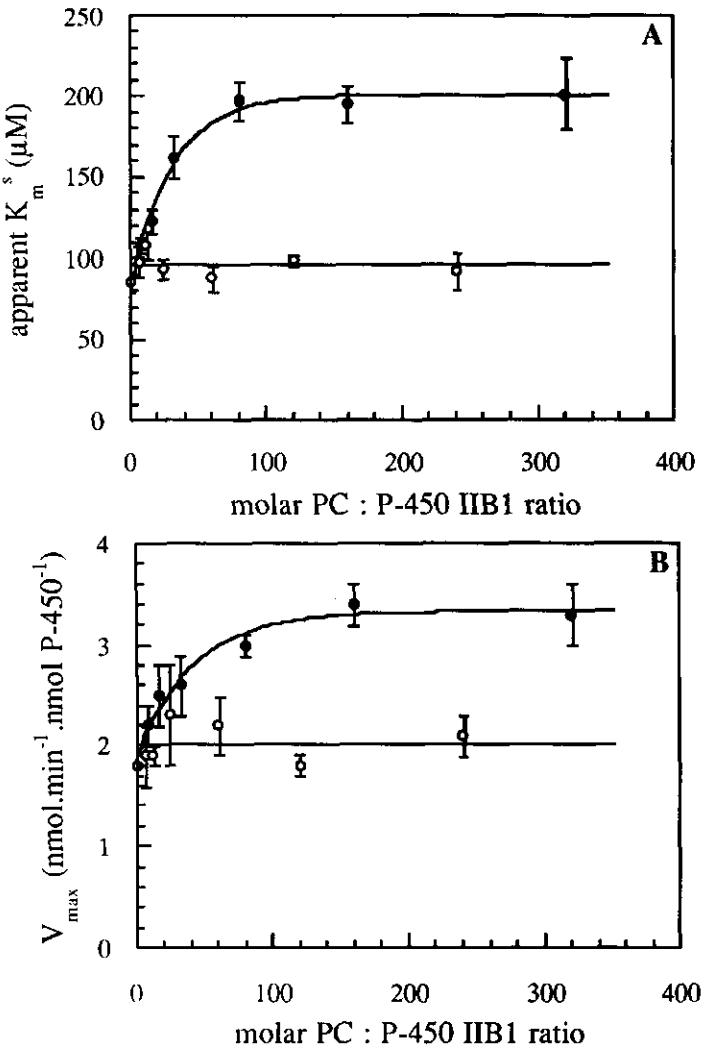

Figure 3.2: The effect of changes in the PC / P450 ratio on the K_m^s (A) and V_{max} (B) of cytochrome P450 IIB1 for ethoxycoumarin in the PC di12:0 (●-●) and PC di18:0 (o-o) systems.

Ethoxycoumarin-O-dealkylating activities were measured as described in 'Materials and methods'. The results represent the mean ± standard error of the mean of two experiments.

In contrast to P450 IA1, an increase in the PC di12:0 : P450 ratio is accompanied by an increase in the K_m^s (Figure 3.2[a]). A similar pattern is observed when the influence of the PC di12:0 : P450 ratio

on the V_{max} of the P450 IIB1 dependent O-dealkylation of ethoxycoumarin was studied (Figure 3.2b). However, an increase in the PC di18:0 : P450 ratio did not affect the K_m^s and V_{max} to a significant extent (Fig. 3.2).

The results above demonstrate that the effects of an increasing phospholipid : P450 ratio on the kinetic parameters of cytochrome P450 IA1 are different from the effects on the kinetic parameters of cytochrome P450 IIB1. Obviously, the mechanism by which phospholipids influence P450 IA1 dependent O-dealkylation of ethoxycoumarin is different from the mechanism by which phospholipids affect the P450 IIB1 dependent reaction.

In a number of additional experiments the mechanism underlying the observed differential effect of PC di12:0 and PC di18:0 on the K_m^s was investigated. The differential effect of PC di12:0 and PC di18:0 on the K_m^s could result from a differential effect of the two PC's on one of the kinetic parameters of the reaction steps of the cytochrome P450 cycle [32]. Kinetic parameters of reaction steps involved in (a) substrate binding, (b) donation of the first electron, (c) oxygen binding, (d) donation of the second electron, (e) reductive oxygen splitting, (f) substrate conversion and / or (g) product release may all influence the K_m^s observed.

3.4.3 Influence of PC di12:0 and PC di18:0 on the K_d^s of cytochrome P450 IA1 and IIB1 for ethoxycoumarin

An effect of the phospholipids on the substrate binding, i.e. on the apparent binding constant of the P450 - substrate complex (K_d^s) (reaction step 1) might be reflected in an influence on the substrate apparent K_m^s. Therefore, the effect of PC di12:0 and PC di18:0 on the K_d^s of the cytochromes P450 for ethoxycoumarin was determined. Cytochrome P450, NADPH-cytochrome reductase and phospholipids were preincubated at the molar ratios used in the kinetic experiments to make the results from the kinetic and binding experiments comparable. For cytochrome P450 IA1 no spectral changes were detected upon the addition of ethoxycoumarin. Therefore, the K_d^s of P450 IA1 for ethoxycoumarin could not be determined.

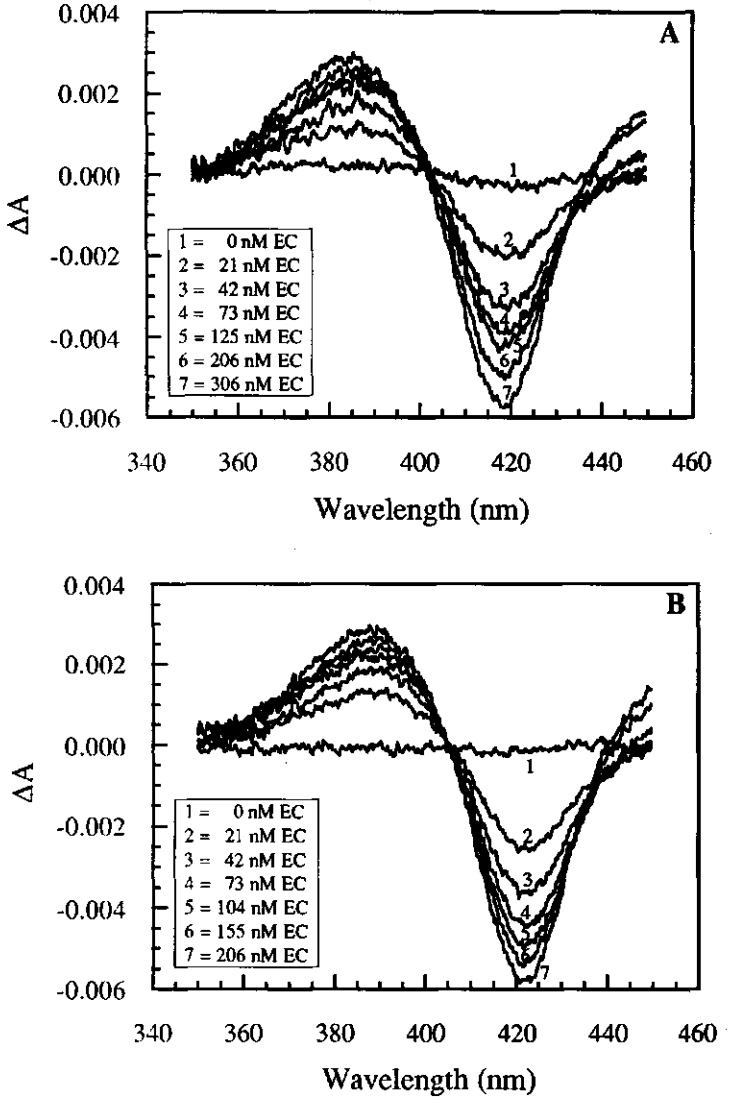

Figure 3.3: Difference spectrum of a purified reconstituted cytochrome P450 IIB1 system upon addition of ethoxycoumarin in the presence of PC di12:0 (A) and PC di18:0 (B).

A solution, containing 1 µM cytochrome P450, 1 µM NADPH-cytochrome reductase and either 320 µM PC di12:0 or 240 µM PC di18:0 was titrated with small amounts of a 10 µM solution of ethoxycoumarin. After each addition the absorption spectrum was recorded between 350 - 450 nm. Further details are described in 'Materials and methods'.

The addition of ethoxycoumarin to a solution containing 1 μM cytochrome P450 IIB1 and 1 μM NADPH-cytochrome reductase in the absence of added PC resulted in a type I difference spectrum. From these difference spectra an ethoxycoumarin apparent binding constant of 57 ± 5 nM was calculated. Preincubation of cytochrome P450 IIB1 and reductase with PC di12:0 and PC di18:0 at PC / P450 ratios of 320 and 240, respectively, resulted in an increase in the high spin content of cytochrome P450 (data to be published elsewhere). Subsequent addition of ethoxycoumarin further increased the high spin content (Fig. 3.3, [a] and [b]). From these results K_d^s values of 39 ± 2 nM and 25 ± 1 nM were calculated for a reconstituted P450 IIB1 system in the presence of PC di12:0 and PC di18:0 respectively. Comparison of these results to the data presented in Table 3.2 demonstrates that the K_m^s value is at least 1000-fold higher than the values obtained for the K_d^s. From this result it can be concluded that, although the K_d^s value of P450 IIB1 for ethoxycoumarin is different in the presence of PC di12:0 from that in the presence of PC di18:0, this parameter is not the main factor influencing K_m^s. Therefore the different effects of PC di12:0 and PC di18:0 on the K_m^s must originate from effects on other kinetic parameters of the P450 reaction cycle.

3.4.4 Influence of the oxygen concentration on the kinetics of O-dealkylation of ethoxycoumarin by P450 IA1 and IIB1

The differences in K_m^s in the presence of PC di12:0 and PC di18:0 of P450 IA1 or IIB1 for ethoxycoumarin could result from a difference in the effect of PC di12:0 and PC di18:0 on the availability of oxygen for the two cytochrome P450 enzymes (reaction step 3). To determine whether the oxygen concentration was rate limiting at the conditions in the experiments described above, the effect of PC di12:0 and PC di18:0 on the kinetic parameters of P450 dependent O-dealkylation of ethoxycoumarin was also investigated at increased oxygen concentrations. To obtain increased oxygen concentrations, the reaction mixture was flushed for 1 min. with oxygen, prior to the addition of NADPH. The results from these experiments demonstrated that an increase in the oxygen concentration has no effect on the kinetic parameters of the O-dealkylation of ethoxycoumarin (data not shown).

3.4.5 Influence of electron donation on the kinetics of O-dealkylation of ethoxycoumarin by P450 IA1 and IIB1

The differential effect of PC di12:0 and PC di18:0 on the K_m^s of P450 IA1 and IIB1 for alkoxyresorufins and ethoxycoumarin may also originate from a different effect of the phospholipids on the interaction of NADPH-cytochrome reductase to cytochrome P450 influencing the rate of electron transfer (reaction step 2 and 4). To investigate this possibility the kinetic experiments were repeated at a reductase / P450 ratio of 5 instead of 1 used in the experiments described above. The results, summarized in Table 3.3, demonstrate that an increase in the reductase concentration causes a considerable increase in all V_{max} values of ethoxycoumarin-O-dealkylation. The relative increase in V_{max} was higher in the PC di18:0 than in the PC di12:0 systems, resulting in elimination of the differences in V_{max} in the PC di12:0 and PC di18:0 system at a reductase / P450 ratio of 5 for both P450 enzymes (Table III). However, the different effects of PC di12:0 and PC di18:0 on the K_m^s of cytochromes P450 IA1 and IIB1 for ethoxycoumarin is maintained upon increasing the reductase concentration. Our results are in agreement with those obtained by Miwa and Lu [31] for cytochrome P450 IA1 and IIB1. They observed that upon increasing the reductase / cytochrome P450 ratio from 0.25 to 1 to 4 the K_m^s for ethoxycoumarin was unaffected and only the V_{max} increased. These results demonstrate that the differing effects of the two PC species on the K_m^s for ethoxycoumarin of P450 IA1 and IIB1 cannot be ascribed to their different effects on the kinetic constants of the electron transfer steps in the two reconstituted P450 systems

The identical V_{max} values for the PC di12:0 and PC di18:0 system at high reductase concentration suggest that the about twofold higher V_{max} in the PC di12:0 system, observed in reconstituted systems with a reductase : P450 molar ratio of 1, may result from an improved formation of the P450 / reductase complex compared to the PC di18:0 system. This was confirmed in an additional experiment, demonstrating an about two times lower apparent K_m of cytochrome P450 IIB1 and IA1 for NADPH-cytochrome reductase (K_m^r) in the presence of PC di12:0 compared to PC di18:0 (Table 3.4).

Table 3.3: The influence of the P450 / reductase ratio on the K_m^s and V_{max} of the ethoxycoumarin-O-dealkylation by cytochromes P450 IA1 and IIB1 in the presence of PC di12:0 and PC di18:0.

reconstituted cytochrome P450	phospholipid	Values at P450 / reductase molar ratio of			
		1:1		1:5	
		K_m^s (μM)	V_{max} (nmol.min^{-1} nmol P450^{-1})	K_m^s (μM)	V_{max} (nmol.min^{-1} nmol P450^{-1})
IA1	PC di12:0	28±3	9.2±0.8	27±9	16.5±0.8
	PC di18:0	45±4**	5.6±0.2***	57±6*	14.2±0.7
IIB1	PC di12:0	201±22	3.3±0.3	160±27	9.8±1.9
	PC di18:0	92±11****	2.1±0.2**	65±6*	8.4±1.4

Experiments were essentially carried out as described in 'Materials and methods'. In the case of the high reductase concentration, 0.1 nmol cytochrome P450 and 20 μg phospholipid were incubated for 6 min. with 1.25 U reductase at 37 °C. The results from the kinetic experiments at a reductase / P450 ratio of 5 are the mean ± standard error of the mean of three experiments with two independent P450 and reductase preparations. The results presented for the 1:1 ratio are similar to those in Table 3.1, but are presented again for comparison.

* significantly different from the value for PC di12:0 at P<0.05

** significantly different from the value for PC di12:0 at P<0.01

*** significantly different from the value for PC di12:0 at P<0.005

**** significantly different from the value for PC di12:0 at P<0.001

From the results described above, the apparent binding constants of the catalytically active P450 / reductase complex in the PC di12:0 and PC di18:0 system can also be determined using the mass action model described by Miwa *et al.* [33]. The calculated K_d^r values are also presented in Table 3.4 and demonstrate that the lower apparent K_m^r value of P450 for reductase in the PC di12:0 system compared to the PC di18:0 system, originates from a lower K_d^r value in the PC di12:0 system. Furthermore, the results show a higher affinity of cytochrome P450 IA1 for NADPH-cytochrome reductase compared to cytochrome P450 IIB1.

Table 3.4: *K_m^r, V_{max} and K_d^r of cytochrome P450 IA1 and IIB1 for NADPH-cytochrome reductase in the presence of either PC di12:0 or PC di18:0.*

reconstituted cytochrome P450	phospholipid	K_m^r (nM)	V_{max} (nmol. min^{-1}. nmol $P450^{-1}$)	K_d^r (nM)
IA1	PC di12:0	10 ± 2	24 ± 2	6 ± 1
	PC di18:0	21 ± 3*	22 ± 2	14 ± 1**
IIB1	PC di12:0	34 ± 3	6 ± 1	28 ± 2
	PC di18:0	70 ± 5**	7 ± 1	63 ± 3**

Ethoxycoumarin-O-dealkylating activities were determined essentially as described in 'Materials and methods'. 0.01 nmol P450 and 2 µg phospholipid was incubated with 0.025-0.25 U NADPH-cytochrome reductase for 6 min. at 37 °C before the ethoxycoumarin-O-dealkylating activity was determined. The apparent K_d^r values were calculated using the mass action model of Miwa *et al.* [33]. The results represent the mean ± standard error of the mean of three experiments.

* significantly different from the value for PC di12:0 at $P<0.05$

** significantly different from the value for PC di12:0 at $P<0.001$

3.5 Discussion

The present study describes the effect of a change in the fatty acid substituents of phosphatidylcholine from dilauroyl to distearoyl on the kinetics of reactions in reconstituted systems dependent on cytochrome P450 IIB1 and IA1. In the reconstituted system used in the present study cytochrome P450 and NADPH-cytochrome reductase were incubated in the presence of phospholipids for 6 min. before activities were determined. This method does not result in incorporation of the proteins into the phospholipid vesicles. Instead, the proteins remain soluble and exist as multimeric protein aggregates with a few individual phospholipids attached to each protein multimer [16,34-36]. The aggregation states of the isolated cytochrome P450 IA1 and IIB1 used in the present study were demonstrated to be comparable [37]. Nevertheless, a different orientation of the two P450 enzymes in their respective multimers might be a factor adding to the

differential effect of phospholipids on the kinetic characteristics of cytochrome P450 IA1 and IIB1 reported here.

The results of the present study demonstrate that PC di12:0 and PC di18:0 differentially affect the kinetic parameters of the reactions dependent on cytochrome P450 IA1 and IIB1. At high phospholipid concentrations (phospholipid / cytochrome P450 ratios of 240 in the PC di18:0 and 320 in the PC di12:0 system) both P450 enzymes exhibited a higher V_{max} in the PC di12:0 system. This higher V_{max} was demonstrated to result from an about two times lower K_m^r and K_d^r of cytochrome P450 for NADPH-cytochrome reductase in the PC di12:0 system. Cytochrome P450 IA1 and IIB1 however, showed an opposite effect of the change from PC di12:0 to PC di18:0 on their K_m^s values. Cytochrome P450 IA1 appeared to have a significantly lower K_m^s in the PC di12:0 system compared with the PC di18:0 system, whereas for P450 IIB1 a significantly higher K_m^s was found in the PC di12:0 system compared to the PC di18:0 system. This was observed not only for the O-dealkylation of pentoxy- and ethoxyresorufin, but also for the O-dealkylation of ethoxycoumarin.

The K_m^s and V_{max} for the P450 IA1 and IIB1 dependent O-dealkylation appeared not only to be dependent on the fatty acyl moiety of the phospholipids but also on the PC / P450 ratio. For cytochrome P450 IA1 an increase in the PC : P450 ratio from 0 to about 300 resulted for both PC species in an exponential decrease in the K_m^s and V_{max}. The K_m^s and V_{max} of P450 IIB1 exhibited a significantly different behaviour. In contrast to P450 IA1, increasing the PC di12:0 / P450 ratio from 0 to about 320 resulted in increases in both the K_m^s and V_{max}, whereas increasing the PC di18:0 concentration did not significantly alter the K_m^s and V_{max}. Especially the decrease in the V_{max} of the reaction catalyzed by cytochrome P450 IA1 upon addition of PC species is surprising compared to the literature data reporting increased substrate conversion rates by reconstituted cytochrome P450 systems upon addition of PC di12:0 [14,19,20,38]. However, in the latter studies conversion rates were generally determined at non-saturating substrate concentrations resulting in conversion rates influenced by V_{max} and K_m^s. Thus, if the decreased V_{max} of P450 IA1 in the presence of PC di12:0 is accompanied by a decreased K_m^s (a situation proved to be the case in the present study) the conversion rate at non-saturating substrate

concentrations might be increased in the presence of PC di12:0 in spite of a decreased V_{max}. From the present results it can be concluded that the stimulating effect of phospholipids on the O-dealkylation of ethoxycoumarin by rat liver P450 IA1 and IIB1 results from an effect on both the K_m^s and V_{max}. Miwa and Lu [19] reported that an increase in the PC di12:0 / P450 ratio from 0 to 480 resulted in an about 8-fold increase in the V_{max} of P450 IIB1 dependent N-demethylation of benzphetamine whereas the K_m^s for benzphetamine was unchanged upon increasing the PC di12:0 / P450 IIB1 ratio. The different observation with respect to the effect of PC di12:0 on the K_m^s of P450 IIB1 reported in the present study is most probably due to the fact that in the study of Miwa and Lu [19] a different substrate was used. Furthermore, from the fact that increasing the PC : P450 ratio from 0 to about 300 results in different effects on the kinetic parameters of O-dealkylation of ethoxycoumarin dependent on cytochrome P450 IA1 and IIB1 (Figs 3.1 and 3.2) it can be concluded that the mechanism by which phospholipids influence P450 IA1 dependent O-dealkylation is different from that by which they influence P450 IIB1 dependent O-dealkylation.

Additional experiments described in the present study were performed to investigate some of the possible reasons for the differential effects of the two PC species on the kinetic parameters of either P450 IA1 or P450 IIB1. The differential effect on K_m^s and V_{max} could result from a different effect of the two PC species on one or more of the many kinetic parameters of the cytochrome P450 cycle . Kinetic parameters of influence could be parameters for (a) substrate binding, (b) transfer of the first electron, (c) oxygen binding, (d) transfer of the second electron, (e) reductive oxygen splitting, (f) substrate conversion and / or (g) product release. In the present study some of these factors were studied in more detail.

The results from cytochrome P450 binding studies demonstrate that the presence of PC di12:0 or PC di18:0 causes a significant decrease in the K_d^s of cytochrome P450 IIB1 for ethoxycoumarin. Nevertheless, it is concluded that the effects of the phospholipids on the K_m^s and V_{max} of P450 IIB1 do not result from an effect on the binding of the substrate (reaction step 1) but from an effect on another rate limiting step of the P450 cycle. This conclusion is derived from the facts that (a) the K_d^s is 1000-fold smaller than the K_m^s and (b) an

increase of the PC / P450 ratio affects the K_m^s and V_{max} in a similar way (Figs 3.1 and 3.2).

In addition, a differential effect of the PC species on the oxygen binding (reaction step 2) cannot be the mechanism underlying the changes observed in V_{max} and K_m^s as an increase in the oxygen concentration did not result in a significant change of the various parameters, demonstrating the oxygen concentration to be optimal at normal saturation.

As a third factor of influence the effect of an increase in the electron flow through the system (steps 2 and 4) was investigated by increasing the reductase / P450 ratio. An increase in the ratio reductase / P450 indeed increased the electron flow through the system resulting in increased V_{max} values for both P450 IA1 and IIB1 (Table 3.3). Additional experiments demonstrated the effect on the V_{max} to be at least in part due to an increased affinity of both cytochromes P450 for the NADPH-cytochrome reductase in the presence of PC di12:0 compared to PC di18:0. This observation is in accord with the increased affinity of $P450_{PB\text{-}B}$ (IIB1) and $P450_{bNF\text{-}B}$ (IA1) for reductase in the presence of PC di12:0 compared to these systems in the absence of PC [19,20]. Furthermore, although the absolute values for K_m^r and K_d^r for the reductase reported in the present study in the presence of PC di12:0 differ somewhat from the ones reported by Müller-Enoch *et al.* [20] (also using enzymes from rat liver), the present observation of a higher reductase affinity of P450 IA1 than of P450 IIB1 was consistent in both studies. The higher affinity of both P450 enzymes for reductase is however, not in agreement with results published before, demonstrating a similar effect of PC di12:0 and PC di18:0 on the reductase K_m^r and K_d^r of cytochrome P450 IIB1 for the conversion of pentoxyresorufin [16]. Because substrates are known to affect the formation of the catalytically active P450 / reductase complex [35], this different observation by Rietjens *et al.* [16] may be ascribed to the fact that the apparent K_m^r and K_d^r of cytochrome P450 for reductase were determined in the presence of pentoxyresorufin instead of ethoxycoumarin as was done in the present study.

The results of the experiments of the present study with increasing reductase : P450 ratio also demonstrated that the disappearence of the difference in V_{max} in the presence of PC di12:0

or PC di18:0 was not accompanied by the disappearance of the differences in the effect of both PC species on the K_m^s for ethoxycoumarin. At increased electron flow through the system the K_m^s of P450 IA1 for ethoxycoumarin is still lower in the presence of PC di12:0 than in the presence of PC di18:0 whereas the K_m^s of P450 IIB1 for ethoxycoumarin is still higher in the presence of PC di12:0 than in the presence of PC di18:0. From this observation it is concluded that although PC species influence the electron flow through the system, this is not the factor causing the differential effect of PC di12:0 and PC di18:0 on the K_m^s of P450 IIB1 and IA1 for ethoxycoumarin. Altogether this implies that the best hypothesis for future research is that the different effect of the phospholipids on the K_m^s of P450 IA1 and IIB1 results from an influence on the kinetic parameters of reductive oxygen splitting, substrate conversion and / or product release.

Because the effects of increasing the PC : P450 ratio on the K_m^s and V_{max} for ethoxycoumarin were similar (Figures 3.1 and 3.2) but differ for P450 IA1 and IIB1, it is concluded that this additional kinetic parameter influencing K_m^s also influences V_{max}. The factor causes a decrease in K_m^s and V_{max} with added PC for P450 IA1, especially with PC di18:0, and an increase in K_m^s and V_{max} with addition of PC di12:0 to P450 IIB1, PC di18:0 having no effect.

In conclusion, results of the present study using reconstituted systems with cytochrome P450 IA1 and IIB1, NADPH-cytochrome reductase and PC di12:0 or PC di18:0 demonstrate an influence of the two model phospholipids on (a) substrate binding to cytochrome P450, (b) affinities of cytochromes P450 for NADPH-cytochrome reductase and thus on (c) the electron flow through the reconstituted system and on (d) the apparent K_m^s of the two cytochromes P450 for a similar substrate. The latter effect must be related to an effect of PC species on kinetic parameters of either the reductive oxygen splitting , substrate conversion and / or product release.

The results also demonstrate that the apparent K_m^s , K_d^s and Vmax values of cytochromes P450 for the substrate and K_m^r and K_d^r of cytochromes P450 for NADPH-cytochrome reductase are dependent on (a) the fatty acyl moiety of the PC, (b) the ratio PC / P450 used and (c) the ratio reductase / P450. In contrast to what has been generally accepted, based on results from conversion rate

measurements under non-saturating substrate conditions [14,19,20,38], the present study demonstrates that the addition of PC species might even result in lower conversion rates (V_{max}) of a reconstituted system. However, this is not reflected in lower but in higher conversion rates at non-saturating substrate concentrations due to a simultaneously lowered K_ms. Furthermore, the effects on the kinetic parameters, observed upon changing the type of PC and / or the PC : P450 ratio appear to be dependent on the P450 enzyme used in the reconstitution. This has not been as well recognized in studies reported up to now.

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Chapter 4

The effect of variation in the headgroup and fatty acyl chains of phospholipids on their binding to cytochrome P450 IIB1

4.1 Summary

In the present study the interaction of cytochrome P450 IIB1 with phospholipids with varying headgroups or fatty acyl chains was investigated. The apparent dissociation constant (K_d) of the cytochrome P450 IIB1 - phospholipid complex was determined for various phospholipids.

All phospholipids studied induced a Type I difference spectrum upon addition to purified cytochrome P450 IIB1. The apparent K_d for the cytochrome P450 IIB1 - phospholipid complex, determined from these binding spectra, appeared to be independent of the length of the fatty acid chains of the phospholipid molecule. In contrast, the apparent K_d of cytochrome P450 IIB1 for the different PC's studied shows a tendency to decrease with increasing degree of desaturation of the fatty acyl moiety of this type of phospholipid. Furthermore, the apparent K_d of cytochrome P450 IIB1 for phospholipids was dependent on the headgroup of the phospholipid, demonstrating a significantly higher apparent K_d of cytochrome P450 IIB1 for PE di16:0 compared to the apparent K_d values observed with PS di16:0, PC di16:0 and PI 16:0/18:1.

Together the data demonstrate that the affinity of cytochrome P450 IIB1 for different phospholipids can be influenced by the headgroup and the degree of desaturation of the fatty acyl chains of a phospholipid. The length of the acyl chains is of minor or no importance.

4.2 Introduction

The hepatic cytochrome P450 is the terminal monooxygenase of an electron transport chain which is capable of catalyzing a wide variety of different reactions. This enzyme system plays a key role in the metabolism of drugs, steroids and other xenobiotics. It is well known that besides cytochromes P450 and NADPH-cytochrome reductase a third component, namely phospholipid is needed for optimal activity [1-4]. Furthermore, it was demonstrated that the dilauroyl derivative of phosphatidylcholine (PC di12:0) was very effective in stimulating cytochrome P450 dependent reactions in reconstituted systems [1-4].

Since then the role of phospholipids in the cytochrome P450 enzyme system has been a topic for many investigations. For the mitochondrial cytochrome P-450_{scc} it was demonstrated that the specific requirement of this P450 enzyme for cardiolipin [5] resulted from a specific effector site on this mitochondrial cytochrome P450 for cardiolipin [6]. For microsomal cytochromes P450 the existence of specific lipid-protein interactions has also been investigated. Already in 1973 Stier and Sackmann [7] suggested that in rabbit liver microsomes the cytochrome P450 / NADPH-cytochrome reductase complex is enclosed by a halo of phospholpids which differs from the bulk phospholipid matrix. More recently, Bayerl *et al.* [8] reported a preference of liver microsomal cytochrome P450 of phenobarbital pretreated rabbits for the binding of PE, demonstrated by use of ^{31}P-NMR, quasi-electric light scattering and freeze-fracture electron microscopy. Bösterling *et al.* [9] studying cytochrome P450 LM_2 - phospholipid interactions in reconstituted vesicles reported a preference for the negatively charged phospholipid phosphatidic acid (PA). A special role for negatively charged phospholipids was also observed by Ingelman-Sundberg *et al.* [10] who demonstrated that a phospholipid mixture of phosphatidyl ethanolamine (PE) and the negatively charged phosphatidylserine (PS) could stimulate cytochrome P450 dependent substrate conversions even further than PC di12:0. Despite these observations, the existence of boundary phospholipids for the microsomal cytochrome P450 system is still poorly established.

The objective of the present study was to investigate the interaction of a cytochrome P450 enzyme with phospholipids with varying headgroup or side-chain. Cytochrome P450 IIB1 from the liver of phenobarbital pretreated rats was used as the P450 protein. The phospholipids used in the present study were PC di10:0, PC di12:0, PC di14:0, PC di16:0, PC di18:0 (increasing length of the side chain), PC di18:1, PC di18:2, PC di20:4 (increasing degree of desaturation), PE di16:0, PS di16:0 and PI di16:0/18:1 (varying headgroup). Possible influences of a variation in the headgroup or the side-chains of a phospholipid on its interaction with a cytochrome P450 form have not been systematically studied before. The apparent dissociation constant (K_d) of the cytochrome P450 IIB1 - phospholipid complex was determined on the basis of the type I

binding spectrum of purified cytochrome P450 IIB1 induced by the various phospholipids.

4.3 Materials and methods

4.3.1 Preparation of microsomes

Microsomes were prepared from the perfused livers of male Wistar rats which were pretreated with phenobarbital (Brocacef b.v., Maarssen, The Netherlands) (0.1% in drinking water). Following homogenization of the livers in Tris/sucrose buffer (50 mM Tris , 0.25 M sucrose pH 7.4) and centrifugation at 10,000 x g (20 min), the supernatants were centrifuged for 75 minutes at 105,000 x g. The microsomal pellet was suspended in 0.1 M potassium phosphate pH 7.25, containing 20% glycerol and 0.1 mM EDTA, immediately frozen into liquid nitrogen and stored at -90 °C until use for purification of cytochrome P450 IIB1.

4.3.2 Purification of cytochrome P450 IIB1

Cytochrome P450 IIB1 was isolated from liver microsomes from male Wistar rats, pretreated with phenobarbital, essentially as described by West *et al.* [11]. Excess detergent was removed by additional washing of the hydroxylapatite column bound protein, using the prescribed buffers without detergents, after which the protein was eluted with 0.3 M potassium phosphate, pH 7.25 containing 20% glycerol. The final detergent-free preparation contained 14 nmoles of P450 heme/mg protein. The cytochrome P450 preparation was over 90% pure as judged by SDS-PAGE [12] and identified as cytochrome P450 IIB1 in an immunoblotting experiment using the monoclonal antibodies described by Letawe-Goujon *et al.* [13]. The concentration of cytochrome P450 was determined from the CO-difference spectrum of the dithionite-reduced protein, based on an extinction coefficient of 91 $mM^{-1}.cm^{-1}$ as described by Omura and Sato [14]. Protein concentrations were measured using the method of Lowry *et al.* [15] using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard.

4.3.3 Determination of the apparent dissociation constant (K_d) of cytochrome P450 IIB1 for various phospholipids

The apparent K_d values were obtained from the phospholipid induced spectral shift in the spin equilibrium of cytochrome P450. All phospholipids were purchased from Sigma (St.Louis, MO, USA). The apparent K_d values were determined by the stepwise addition of a phospholipid suspension (0.01 mg/ml in water) to 1 μM of cytochrome P450 in 0.1 M potassium phosphate buffer (pH 7.5) containing 20 % glycerol. After each addition the absorption spectrum between 350 and 450 nm was recorded against a blank cuvet filled with buffer to which similar stepwise additions of phospholipid were made. The spectra were corrected for the absorption spectrum of cytochrome P450 IIB1 without added phospholipid measured against the buffer. The absorption spectra were recorded on an SLM/Aminco DW-2000 spectrofotometer at 25 °C. The apparent K_d values were calculated by fitting the data to the function:

$$\Delta A = \frac{\Delta A_{max} * [S]}{K_d + [S]}$$

where ΔA_{max} = maximum $\Delta A_{(386\text{-}420)}$

$[S]$ = phospholipid concentration

ΔA = $\Delta A_{(386\text{-}420)}$ at a certain phospholipid concentration

K_d = apparent dissociation constant for the P450 IIB1-phospholipid complex.

4.3.4 Statistical analysis of data

The results presented are the mean ± standard error of the mean. Statistical analysis was carried out using Student's t-test, for unpaired samples. The null-hypothesis was rejected at $P \leq 0.05$.

4.4 Results

4.4.1 The effect of the length and the degree of desaturation of the fatty acyl chains on the apparent dissociation constant of the cytochrome P450 IIB1 - phosphatidylcholine complex

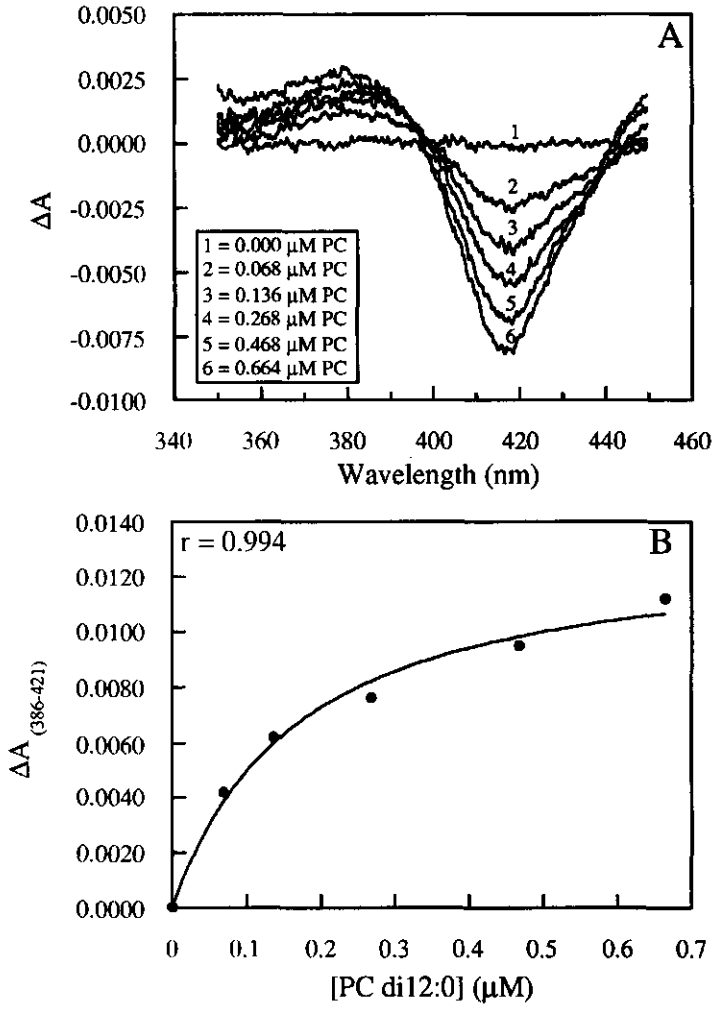

Figure 4.1: Difference spectra of purified cytochrome P450 IIB1 upon addition of PC di12:0 (A) and calculation of the apparent K_d value from the difference spectra (B).

A solution, containing 1 µM cytochrome P450 IIB1 in 0.1 M potassium phosphate (pH 7.5) containing 20% glycerol was titrated with small amounts of a 16 µM solution of PC di12:0. After each addition the absorption spectrum was recorded between 350 and 450 nm. Further details are described in 'Materials and methods'. The apparent K_d value was calculated by plotting $\Delta A_{(386-421)}$ obtained from Figure 1[a] as a function of the phospholipid concentration. The plotted line presents the best fit of the data to the function $\Delta A = \frac{\Delta A_{max} * [S]}{K_d + [S]}$. The correlation coefficient was 0.994.

In Figure 1[a] the difference spectra of purified cytochrome P450 IIB1 obtained upon the stepwise addition of PC di12:0 are presented. These results demonstrate that the addition of PC di12:0 to cytochrome P450 IIB1 induces a type I difference spectrum with a peak at 386 nm and a through at 421 nm, typical of a shift in the spin equilibrium of the cytochrome P450 from low spin to high spin. Type I binding spectra were also observed upon the addition of the other phospholipids (data not shown). Figure 1[b] presents the plot of the PC di12:0 concentration versus the $\Delta A_{(386-420)}$ derived from the a difference spectrum. For all phospholipids the ΔA_{max} was comparable (average $\Delta A_{max} = 0.0214 \pm 0.0008$).From this plot an apparent K_d value of 0.164 μM was calculated. The apparent K_d values of cytochrome P450 IIB1 for the other phosphatidylcholine species were obtained in a similar way. The apparent K_d of cytochrome P450 IIB1 was determined for PC species with varying length and degree of desaturation of the fatty acyl chains.

Table 4.1: Determination of the effect of the fatty acyl chain length on the apparent K_d values of cytochrome P450 IIB1 for PC.

phospholipid	apparent K_d (μM)
PC di10:0	0.142 ± 0.009
PC di12:0	0.155 ± 0.020
PC di14:0	0.148 ± 0.010
PC di16:0	0.144 ± 0.022
PC di18:0	0.152 ± 0.020

The apparent dissociation constants were determined as described in 'Materials and methods'. The results represent the mean ± standard error of the mean of 3 experiments.

The results, presented in Tables 4.1 and 4.2, demonstrate that the apparent K_d value of cytochrome P450 IIB1 for PC is independent of the length of the fatty acyl moieties (Table 4.1). However, upon increasing the degree of desaturation of the fatty acid chains the apparent K_d shows a tendency to decrease. The apparent K_d of P450 IIB1 for PC di20:4 is even significantly ($P<0.05$) smaller that the apparent K_d for PC di18:0 (Table 4.2). Expression of the apparent K_d values as a function of the number of unsaturated bonds per fatty acyl chain results in a linear relationship with a correlation coefficient of

0.996 and a decrease of about 0.007 μM in the apparent K_d value for an additional bond in the fatty acid moiety of the phospholipid molecule.

Table 4.2: Determination of the effect of degree of desaturation of the fatty acyl chain on the apparent K_d values of cytochrome P450 IIB1 for PC.

phospholipid	apparent K_d (μM)
PC di18:0	0.152 ± 0.020
PC di18:1	0.134 ± 0.001
PC di18:2	0.125 ± 0.008
PC di20:4	0.094 ± 0.011*

The apparent dissociation constants were determined as described in 'Materials and methods'. The results represent the mean ± standard error of the mean of 3 experiments. The value for PC di18:0 is similar to the one given in Table I but presented again for comparison.

* significantly different from the value presented for PC di18:0 at $P<0.05$

4.4.2 The effect of the phospholipid headgroup on the apparent K_d of the cytochrome P450 IIB1 - phospholipid complex

In addition to the fatty acyl chain, the binding of phospholipids to cytochrome P450 might also be dependent on the headgroup of the phospholipids. Therefore, the apparent K_d of cytochrome P450 IIB1 for three other major phospholipid constituents of the endoplasmic membrane besides PC, namely PE, PI and PS were also determined. The model phospholipids chosen contained two palmitoyl side chains (16:0) if possible. Because PI di16:0 is not commercially available PI 16:0/18:1 was chosen for studying the effect of a inositol headgroup. Based on the results presented above, replacement one 16:0 acyl chain by a 18:1 acyl chain is expected to influence the K_d only to a very limited extend, i.e. an estimated decrease by about 0.007 μM. Again, all phospholipids studied induced type I difference spectra . The apparent K_d values, calculated from these cytochrome P450 difference spectra, are presented in Table 4.3. The data demonstrate that the headgroup of the phospholipids can affect the apparent K_d of the cytochrome P450 IIB1 - phospholipid complex. The apparent K_d for

PE is significantly higher than the apparent K_d value for PC, PS and PI. The values for PC, PI and PS do not differ significantly.

Table 4.3: Determination of the effect of the phospholipid headgroup on the apparent K_d values of cytochrome P450 IIB1 for phospholipids.

phospholipid	apparent K_d (μM)
PC di16:0	0.144 ± 0.022
PE di16:0	0.238 ± 0.016*
PS di16:0	0.131 ± 0.015
PI 16:0/18:1	0.141 ± 0.013

The apparent dissociation constants were determined as described in 'Materials and Methods'. The results represent the mean ± standard error of the mean of 6 experiments. The value for PC di16:0 is similar to the one given in Table I but presented again for comparison.

* significantly different from the value for PC ($P<0.02$), PIno ($P<0.002$) and PSer ($P<0.001$).

4.5 Discussion

In the present study the effect of the headgroup and fatty acyl moiety of phospholipids on their binding affinity to cytochrome P450 IIB1 is investigated. The apparent K_d values were obtained from the phospholipid induced type I shift in the spin equilibrium of cytochrome P450 IIB1. Using PC's with varying length and degree of desaturation of the fatty acyl chains it could be demonstrated that the apparent K_d of the cytochrome P450 IIB1 - phospholipid complex is not dependent on the length of the fatty acid chains. However, a tendency was observed for the apparent K_d to decrease with an increase in the number of double bonds in the fatty acyl side chains of phosphatidylcholine. Furthermore, the apparent K_d appeared to be dependent on the headgroup of the phospholipid. PS di16:0, PC di16:0 and PI 16:0/18:1 were demonstrated to have similar apparent K_d values. PE di16:0 however, appeared to have a significantly higher apparent K_d value.

The apparent K_d values obtained in the present study are three orders of magnitude smaller than the apparent K_d value of 0.45 mM

reported by Chiang and Coon [16] for the rabbit cytochrome P450 LM_2 - PC di12:0 complex. French *et al.* [17] observed a biphasic behaviour for the binding of PC di12:0 to cytochrome P450 LM_2 representing binding with high and low affinity. The apparent dissociation constants were estimated to be 3-6 μM and 50-70 μM for respectively the high and low affinity site. Although smaller than the apparent K_d value reported by Chiang and Coon [16], the values reported by French *et al.* [17] are still 1 to 2 orders in magnitude higher than the apparent K_d of cytochrome P450 IIB1 for PC di12:0 observed in the present study. These different observations for the apparent K_d for the P450 IIB1 - PC di12:0 complex may be ascribed to the facts that (i) in the studies of Chiang and Coon [16] and French *et al.* [17] rabbit cytochrome P450 IIB1 was used whereas in this study rat cytochrome P450 IIB1 was investigated and / or (ii) the different (more extensive) method for the removal of detergent from the isolated cytochrome P450 IIB1 preparation used in the present study resulting in the liberation of high affinity phospholipid binding sites.

Up to now, the information on the existence of specific lipid-protein interactions in the cytochrome P450 enzyme system is limited. Bösterling *et al.* [9] reported a preference of reconstituted cytochrome P450 LM_2 for the negatively charged phospholipid PA. The basis for specific PA / P450 interactions would be the charge-charge coupling of positive charges on the protein surface and the negative charge on the phospholipid headgroup. In analogy to this observation the higher apparent K_d of the uncharged PE di16:0 compared to the apparent K_d values of the negatively charged PI 16:0/18:1 and PS di16:0 might be ascribed to the absence of electrostatic interactions between PE and cytochrome P450 IIB1 resulting in a lower affinity i.e. a higher apparent K_d. However, the significantly lower apparent K_d of the uncharged PC di16:0 compared to PE di16:0 must result from a different effect.

Bayerl *et al.* [8] reported a preference of microsomal cytochrome P450 from phenobarbital pretreated rabbits for the binding of PE. This preference of cytochrome P450 for PE was suggested to result from the shape of this phospholipid [18]. The relatively small headgroup of PE compared to the fatty acyl moiety gives this phospholipid a wedge shape which was suggested to adapt

cytochrome P450 to the membrane [18]. The results of the present study demonstrate that the apparent K_d of cytochrome P450 IIB1 for the wedge shaped PE is significantly higher than the apparent K_d's for the cilindrically shaped (phospholipid headgroup and fatty acyl chain are about equal in size) phospholipids PC, PI and PS. On the other hand, the results also show that increasing the degree of desaturation of the fatty acyl chain, which increases the size of the fatty acyl moiety relative to the headgroup and thus can be expected to make the molecule more wedge shaped, results in a significant decrease in the phosphatidylcholine apparent K_d. From this it is concluded that the shape of the phospholipid molecule is not factor determining the affinity of cytochrome P450 IIB1 for phospholipids and that the interaction of phospholipids with cytochromes P450 is more likely to be dependent on a combination of electrostatic and hydrophobic interactions.

In conclusion, the results of the present study demonstrate that the affinity of cytochrome P450 IIB1 for different phospholipids is dependent on both the headgroup as well as the degree of desaturation of the fatty acyl moiety of a phospholipid molecule. However, in the present study the dependency on fatty acid chain length and degree of desaturation was characterized for PC phospholipids. For a further understanding of the factors determining the interaction of phospholipids with cytochrome P450, the effect of chain length and degree of desaturation of phospholipids with other headgroups on the apparent K_d might have to be investigated. Because series of pure molecular species of PE, PS and PI with varying fatty acyl chain characteristics are not commercially available these studies have to await synthesis and / or purification of the model phospholipids. Furthermore it remains to be established whether the factors that were shown to influence cytochrome P450 IIB1 - phospholipid interactions in the solublized system also play a role in the microsomal membrane.

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Chapter 5

A specific interaction between NADPH-cytochrome reductase and phosphatidylserine and phosphatidylinositol

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5.1 Summary

In the present study the interaction of NADPH-cytochrome reductase with phospholipids was investigated using ^{31}P-NMR, thin layer chromatography combined with chemical analysis, fluorescence spectroscopy and kinetic studies with purified rat liver cytochrome P450 IIB1.

^{31}P-NMR analysis demonstrates that the composition of the phospholipids that remain associated to NADPH-cytochrome reductase upon its purification is significantly different from the phospholipid composition of the microsomal membrane. thin layer chromatography followed by chemical analysis of the phospholipid composition demonstrates that the isolated NADPH-cytochrome reductase was enriched in L-α-1,2-diacyl-*sn*-glycero-3-phosphoserine (PS) and L-α-1,2-diacyl-*sn*-glycero-3-phosphoinositol (PI) compared to the microsomal membrane. The observed preference of NADPH-cytochrome reductase for PS and PI appeared not to be a result of the procedure for solubilisation and / or purification of the protein.

The specific interaction of NADPH-cytochrome reductase with PS and PI was further investigated by comparison of the effect of PS and PI with that of PC and PE on the 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (DPH-PC) dependent quenching of the tryptophan fluorescence of purified NADPH-cytochrome reductase. The results demonstrate that the addition of PS or PI affects the DPH-PC dependent quenching of the tryptophan fluorescence in a manner significantly different from the addition of PC or PE. The relatively larger DPH-PC induced quenching of the tryptophan fluorescence of NADPH-cytochrome reductase in the presence of PS and PI must result from a PS and PI induced change in the conformation of NADPH-cytochrome reductase.

Finally, the possible consequences of this special interaction of PS and PI with NADPH-cytochrome reductase on the kinetic characteristics of the cytochrome P450 system were studied using cytochrome P450 IIB1 dependent O-dealkylation of pentoxyresorufin as the model reaction. These studies demonstrate that a 1:1 mixture of PC and PS results in a significantly higher apparent maximum rate (V_{max}) of O-dealkylation than a 1:1 mixture of PC and PE or PC alone. This increase in the apparent V_{max} can be ascribed to an PS dependent improvement of

the interaction of NADPH-cytochrome reductase with cytochrome P450. This improvement of the interaction of the proteins can however not be exclusively ascribed to the negative charge of PS, since the other negatively charged phospholipid investigated, namely PI, resulted in a significant decrease in the apparent V_{max}. In both cases the substrate apparent K_m was not affected. This opposite effect of PS and PI on the kinetics of the cytochrome P450 IIB1 system might provide a means for phospholipid mediated regulation of this cytochrome P450 enzyme.

5.2 Introduction

The cytochrome P450 enzyme system is involved in the biotransformation of a wide variety of substrates. The first succesful reconstitution of cytochrome P450 dependent activities already demonstrated the importance of lipid-protein interactions for this membrane bound multi-enzyme complex [1-4]. The interaction of the cytochrome P450 component with phospholipids has been a topic for investigation in several studies. For the mitochondrial cytochrome $P450_{scc}$ a specific requirement was reported for cardiolipin [5] which was later demonstrated to result from a specific effector site on the cytochrome P450 for this phospholipid [6]. For microsomal cytochromes P450 however, the importance of so-called "boundary phospholipids" for the activity of the cytochrome P450 system is still a matter of considerable debate. In 1973 Stier and Sackmann [7] suggested that in rabbit liver microsomes the cytochrome P450 / reductase complex is enclosed by a halo of the phospholipid matrix which differs from the bulk matrix. More recently, Bayerl *et al.* [8] reported a preference of liver microsomal cytochrome P450 of phenobarbital pretreated rabbits for the binding of L-α-1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PE), demonstrated by use of ^{31}P-NMR, quasi-electric light scattering and freeze fracture electron microscopy. The rationale for a specific PE / P450 interaction was suggested to be the wedgeshaped structure of PE which might adapt cytochrome P450 to the bilayer membrane by half-micellar structures [9]. Bösterling *et al.* [10], studying the phospholipid-P450 interactions in reconstituted vesicles, reported a preference of cytochrome P450 LM_2 for the negatively charged phospholipid L-α-1,2-diacyl-*sn*-glycero-3-phosphate (PA). This interaction was suggested to be

based on the charge-charge coupling of the negative charge of the phospholipid headgroup and the positive charges on the surface of the protein. Specific interactions of cytochrome(s) P450 with naturally more abundant negatively charged phospholipids like L-α-1,2-diacyl-*sn*-glycero-3-phosphoserine (PS) and L-α-1,2-diacyl-*sn*-glycero-3-phosphoinositol (PI) might be of more interest. Ingelman-Sundberg *et al.* [11] for example reported an increase in the reconstituted cytochrome P450 LM_2 catalysed O-dealkylation of p-nitroanisole or ethoxycoumarin upon increasing the negative charge of the membrane. Furthermore, an increased thermal denaturation temperature of rat liver microsomal cytochrome P450 was observed in the presence of PI [12]. These data from previous studies point to a special role of negatively charged, naturally-occuring phospholipids in the cytochrome P450 system.

So far, studies on the interaction of the cytochrome P450 system with phospholipids were confined to investigations on the interactions between the cytochrome P450 component and the phospholipids. Very little is known about interactions between phospholipids and NADPH-cytochrome reductase, the other important enzyme component of the cytochrome P450 system. Therefore, the objective of the present study was to investigate the possible specific lipid-protein interactions for NADPH-cytochrome reductase in more detail. This was done using ^{31}P-NMR, chemical analysis, fluorescence spectroscopy and kinetic studies with purified rat liver NADPH-cytochrome reductase and cytochrome P450 IIB1.

5.3 Materials and methods

5.3.1 Preparation of microsomes

Microsomes were prepared from the perfused livers of Wistar rats (±300 g) which were pretreated with phenobarbital (Brocacef b.v., Maarssen, The Netherlands) (0.1% in drinking water, for 7 days). Following homogenisation of the livers in Tris/sucrose buffer (50 mM Tris , 0.25 M sucrose pH 7.4) and centrifugation at 10,000 x g (20 min), the supernatants were centrifuged for 75 minutes at 105,000 x g. The microsomal pellet was resuspended in 0.1 M potassium phosphate pH 7.25, containing 20% glycerol and 0.1 mM EDTA, immediately frozen

into liquid nitrogen and stored at -90 °C until use for isolation of NADPH-cytochrome reductase or cytochrome P450 IIB1.

5.3.2 Chemicals

Phospholipid references, PC, PE, PS, PI, PG, LPC, LPE and LPS were all purchased from Sigma (St. Louis, MO, USA). Emulgen 911 and Lubrol PX used for the purification of NADPH-cytochrome reductase and cytochrome P450 IIB1 were obtained from respectively KAO Chemicals (Tokyo, Japan) and Sigma (St.Louis, MO, USA). 2-(3-(Diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (DPH-PC) was purchased from Molecular Probes Inc. (Eugene, OR, USA).

5.3.3 Purification of proteins

NADPH-cytochrome reductase was isolated from liver microsomes of phenobarbital pretreated Wistar rats essentially as described by Yasukochi and Masters [13]. Emulgen 911 (Kao Corporation, Tokyo, Japan) was used instead of Renex 690, and all buffers contained 100 μM phenylmethanesulfonyl fluoride (Merck, Darmstadt, FRG) to inhibit protease activities. Excess detergent and phospholipid was removed by binding the protein to the 2'5'ADP-sepharose column (15 ml), equilibrated with 20 mM potassium phosphate (pH 7.7) containing 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol (Boehringer, Mannheim, FRG) and 0.1 mM phenylmethanesulfonyl fluoride. The effluent with proteins not bound to the 2'5'ADP-sepharose column was collected, concentrated over an Amicon YM-30 filter and prepared for ^{31}P-NMR as described below. The 2'5'ADP-sepharose column was washed with at least 10 column volumes of equilibration buffer and the NADPH-cytochrome reductase was eluted with the same buffer, containing 10 mM adenosine 2'(3')-monophosphate (Sigma, St.Louis, MO, USA) and 0.2% sodium deoxycholate (Merck, Darmstadt, FRG). The NADPH-cytochrome reductase preparation was concentrated over an Amicon YM-30 filter and dialysed against 3 times 2 litres of 20 mM potassium phosphate (pH 7.7) containing 20% glycerol, 0.1 mM EDTA and 0.1 mM dithiothreitol to remove the deoxycholate and adenosine 2'(3')-monophosphate. The final detergent-free preparation had a specific activity of about 35 units/mg protein and was homogeneous as judged by SDS-PAGE, carried out as described by Laemmli [14].

Cytochrome P450 IIB1 was isolated from liver microsomes of phenobarbital pretreated rats, essentially as described by West *et al.* [15] using Lubrol PX instead of Emulgen 911. Excess detergent was removed by additional washing of the hydroxylapatite column bound protein, using the prescribed buffers without detergents, after which the protein was eluted with 0.3 M potassium phosphate, pH 7.25 containing 20% glycerol. The final detergent-free preparation contained 13 nmoles of P450 heme/ mg protein. The cytochrome P450 IIB1 preparation was over 90% pure as judged by SDS-PAGE and was identified as cytochrome P450 IIB1 in an immunoblotting experiment using the monoclonal antibodies described by Letawe-Goujon *et al.* [16].

5.3.4 Biochemical assays

NADPH-cytochrome reductase activity was determined as described by Phillips and Langdon [17], using cytochrome *c* (Boehringer, Mannheim, FRG) as the final electron acceptor. One µmol cytochrome *c* reducing activity per minute was taken as the unit to quantify the NADPH-cytochrome reductase activity. Protein concentrations were measured using the method of Lowry *et al.* [18] using bovine serum albumin (Sigma, St. Louis, MO,USA) as the standard. The concentration of cytochrome P450 was determined from the CO-difference spectrum of the dithionite-reduced protein, based on an extinction coefficient of 91 $mM^{-1}.cm^{-1}$ as described by Omura and Sato [19].

Lipid extraction from liver microsomes from phenobarbital pretreated rats and from isolated NADPH-cytochrome reductase was carried out according to the method of Bligh and Dyer [20]. The extracted lipids were stored under argon at -20 °C until use. Separation of the lipid mixtures was performed by 1-dimensional thin layer chromatography on prefabricated, 20x20 cm silicagel plates (Sigma, St. Louis, MO, USA) using the solvent chloroform-methanol-acetic acid-water (90:40:12:2). Chromatography was performed at room temperature until the solvent reached the upper edge of the plate (150 minutes). The thin layer chromatography-plate was dried and the lipid spots were visualised by heating the plate after spraying with 20% sulphuric acid in water. The spots on the thin layer chromatography plate were identified using phospholipid references and were assayed for phosphorous content by the method of Rouser *et al.*.[21].

Pentoxyresorufin O-dealkylating activities were measured using the method of Burke *et al.* [22] in 0.1 M potassium phosphate pH 7.5 at 37 °C using 0.25-2 μM of pentoxyresorufin (Boehringer, Mannheim, FRG) added in Me_2SO (10 μl /ml assay). Calibration of the fluorescence measurement for quantification of the resorufin formation was carried out by addition of 10 μl of a 104 μM stock solution of resorufin (Boehringer, Mannheim, FRG) in Me_2SO to the cuvette containing the reaction mixture.

5.3.5 ^{31}P-NMR experiments

The protein preparations were dialysed four times against 1 liter of 50 mM Tris/HCl buffer (pH 7.9), containing 10% glycerol, 5 mM EDTA and 1% sodium cholate (Merck, Darmstadt, FRG) to remove free phosphate. After dialysis the NADPH-cytochrome reductase preparations were concentrated over an Amicon YM-30 filter to a final concentration of approximately 75 μM. The microsomal lipid extract and the phospholipid reference samples were dissolved in 50 mM Tris/HCl buffer (pH 7.9) containing 10% glycerol, 5 mM EDTA and 1% sodium cholate to a final concentration of approximately 2 mM and the solutions were sonicated briefly.

Norell (Landisville, NJ, USA) 10 mm NMR tubes were filled with 1.5 ml sample to which 100 μl ^2H_2O was added for locking the magnetic field. ^{31}P-NMR measurements were performed on a Bruker AMX 300 NMR spectrometer using 30° pulses and a recycle time of 1.1 s. Decoupling of ^{1}H was done with the inverse gated decoupling technique. Chemical shifts were determined relative to an external reference of 85% H_3PO_4 at 303K.

All ^{31}P-NMR samples were measured at 303K except the isolated NADPH-cytochrome reductase, which was measured at 290K. This was done to prevent denaturation of the NADPH-cytochrome reductase during the ^{31}P-NMR experiment.

5.3.6 Solubilisation of liver microsomes of phenobarbital pretreated rats with different (mixtures of) detergents

The liver microsomes of phenobarbital pretreated Wistar rats were diluted with water and glycerol (10% final concentration) to a final protein concentration of 14 mg/ml. Sodium cholate, sodium deoxycholate, Lubrol PX and / or Emulgen 911 were added to the

microsomal solution and the mixture was stirred for 1 hour at 4 °C. After centrifugation at 105,000 x g (75 minutes) the supernatants, containing the solubilised proteins, were concentrated over an Amicon YM-30 filter to approximately 3 ml and dialysed four times against 1 liter of 50 mM Tris/HCl buffer (pH 7.9), containing 10% glycerol, 5 mM EDTA and 1% sodium cholate (Merck, Darmstadt, FRG) to remove free phosphate. The protein preparations were prepared for ^{31}P-NMR as described above.

5.3.7 Quenching of the tryptophan fluorescence of NADPH-cytochrome reductase

The fluorescence quenching experiments were carried out essentially as described by East and Lee [23]. The lipids were dissolved in 0.1 M Tris/HCl buffer (pH 7.5) containing 3% sodium cholate and the solutions were sonicated briefly. The lipid solutions (final lipid concentration = 0.75 μM) contained different molar fractions of DPH-PC. 0.20 Units of isolated NADPH-cytochrome reductase (± 75 pmol) and 20 nmol of phospholipid in 0.1 M Tris/HCl buffer (pH 7.5) containing 3% sodium cholate were incubated for 30 minutes at 37 °C in a small volume (50 μl). After this preincubation 950 μl of 0.1 M Tris/HCl buffer (pH 7.5) were added and the mixture was allowed to equilibrate for 15 minutes before tryptophan fluorescence was determined. The fluorescence experiments were performed with a SLM/Aminco SPF-500C spectrofluorometer at 37 °C. The excitation wavelength was 280 nm and tryptophan fluorescence was detected at an emission wavelength of 340 nm. The presence of sodium cholate (less than 0.1% in the final mixture) which was used to dissolve the phospholipids did not affect the tryptophan fluorescence.

5.3.8 Reconstituted incubations

To determine the effect of phospholipids with different headgroups on the kinetics of the cytochrome P450 IIB1 dependent O-dealkylation of pentoxyresorufin 0.04 nmol of cytochrome P450, 0.1 units of NADPH-cytochrome reductase and 10 nmol of phospholipid (resulting in a molar P450 : reductase : phospholipid ratio of 1:1: 250) were preincubated in a small volume (50 μl) for 6 minutes at 37 °C before buffers, substrate and NADPH (0.4 μM final concentration) were added up to a volume of 1 ml. This type of reconstitution does not result in incorporation of the proteins into the phospholipid vesicle. Instead, the proteins remain soluble and

exist as homomultimeric protein aggregates with a few individual phospholipids attached to each protein multimer [24-27].

The O-dealkylating activity was determined as described above with a SLM/Aminco SPF-500C spectrofluorometer at 37 °C and is expressed as nmol resorufin formed. min^{-1}. nmol $P450^{-1}$.

5.3.9 Statistical analysis of data

The results are presented as the mean ± standard error of the mean (SEM). Statistical analysis was carried out using Students' t-test, for unpaired samples. The null-hypothesis was rejected at $P \leq 0.05$.

5.4 Results

5.4.1 ^{31}P-NMR analysis of purified NADPH-cytochrome reductase

^{31}P-NMR was used to investigate the composition of the phospholipids that remain associated to NADPH-cytochrome reductase upon its purification. Figure 5.1[a] presents the part of the ^{31}P-NMR spectrum containing the ^{31}P-NMR resonances of reductase-bound phospholipids [28-30]. Comparison of the ^{31}P-NMR spectrum of isolated NADPH-cytochrome reductase with that of the phospholipid extract of the microsomal membrane (Figure 5.1[b]) demonstrates that the phospholipid content of the purified NADPH-cytochrome reductase differs significantly from that of the microsomal extract. Comparison of ^{31}P-NMR data of three independent NADPH-cytochrome reductase preparations demonstrated that, compared to the microsomal lipids, the NADPH-cytochrome reductase preparations show relatively higher intensities of the peaks at 0.16, 0.48 and 1.00 ppm, whereas the intensities at 0.02 and 0.66 ppm are relatively lower. Unequivocal identification of the various phospholipid peaks in the ^{31}P-NMR spectrum of the isolated NADPH-cytochrome reductase preparations was hampered by the small differences in chemical shift of PS (0.52 ppm) and LPC (0.50 ppm) as well as of LPE (1.01 ppm) and PG (1.05 ppm), in combination with the relatively broad line width of the peaks.

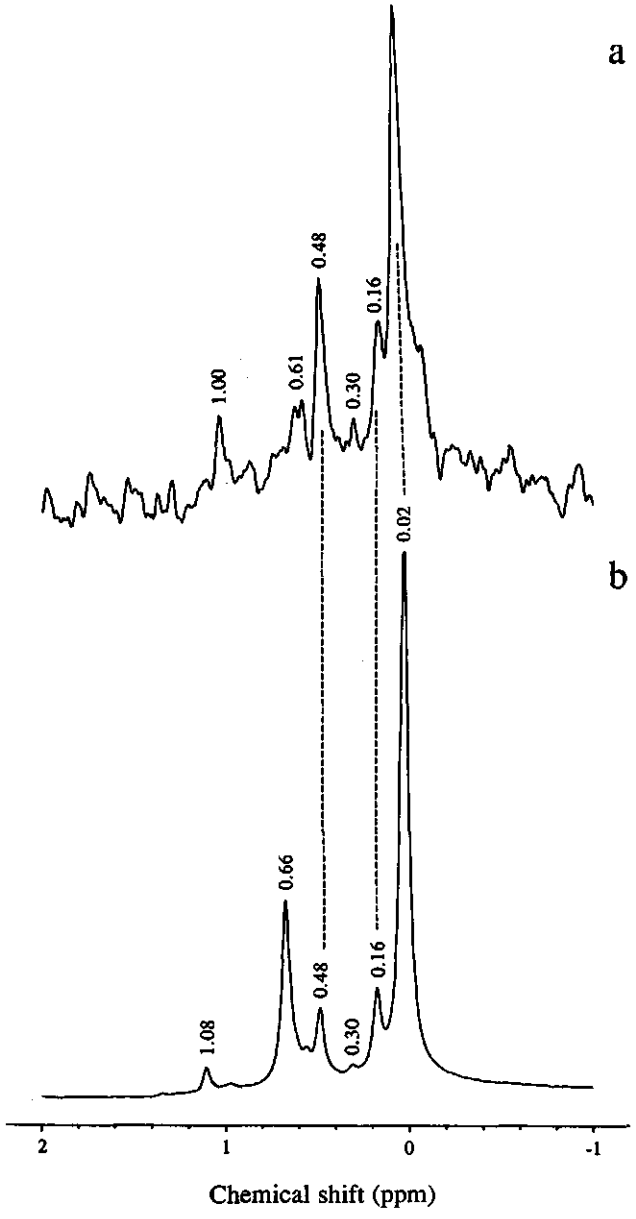

Figure 5.1: *^{31}P-NMR spectra of isolated NADPH-cytochrome reductase and a phospholipid extract of microsomes of phenobarbital pretreated rats.*
Isolated NADPH-cytochrome reductase (a) and the phospholipid extract (b) were prepared for ^{31}P-NMR as described in 'Materials and methods'.

Nevertheless, comparison of the chemical shift values of the reference phospholipids (Table 5.1) to the ^{31}P-NMR spectra presented in Figure 5.1 indicates that the peaks at 0.02, 0.16 and 0.61 ppm probably belong to respectively PC, PI and PE. The peak at 0.48 ppm could either be LPC or PS. Results from thin layer chromatography-experiments however, described hereafter, demonstrate that the peak at 0.48 ppm belongs to PS, since no LPC spot was observed on the thin layer chromatography plate. Thus, these results point to a relative enrichement of the isolated NADPH-cytochrome reductase in PI and PS.

Table 5.1: ^{31}P-NMR chemical shift of phospholipids in 50 mM Tris/HCl buffer (pH 7.9) containing 10% glycerol, 5 mM EDTA and 1% cholate at 303 K.

phospholipid	chemical shift (ppm)
PC	0.01
PI	0.16
LPC	0.50
PS	0.52
PE	0.61
LPS	0.82
LPE	1.01
PG	1.05

The phospholipid solutions were prepared for ^{31}P-NMR as described in 'Materials and methods'.

5.4.2 Characterisation and quantification of the phospholipid contents of the purified NADPH-cytochrome reductase by thin layer chromatography and chemical analysis

Because the ^{31}P-NMR data presented above only allowed qualitative analysis and comparison of the phospholipid content of isolated reductase to that of the microsomal membrane, the phospholipid compositions of the isolated NADPH-cytochrome reductase preparation and the microsomal membrane were also determined by thin layer chromatography in combination with chemical analysis. This method is much more sensitive than ^{31}P-NMR and allows a better resolution of LPC, PS, PE and SM, although separation of PS and PI cannot be achieved [31]. The individual lipid spots on the thin layer

chromatography plate were assayed for phosphorous content and the results from these chemical analyses are presented in Table 5.2. For comparison, Table 5.2 also presents data reported in the literature for the phospholipid composition of the microsomal membrane. Finally, Table 5.2 presents a phospholipid composition of the microsomal membrane lipid extract as derived from its ^{31}P-NMR spectrum already presented in Figure 5.1[b].

Table 5.2: The phospholipid composition of rat liver microsomes of phenobarbital pretreated rats and isolated NADPH-cytochrome reductase preparations.

Preparation	Phospholipid composition (% of total)					
Method	PA	PC	PE	SM	PS+PI ‡	PG
microsomes						
TLC + chemical analysis	1 ± 1	57 ± 1	26 ± 2	2 ± 1	15 ± 1	n.o.
^{31}P-NMR	n.o.	59	21	n.o.	7 ± 10	3
literature [32]	n.o.	62	23	2	14	n.o.
isolated reductase						
TLC + chemical analysis	n.o.	57 ± 3	20 ± 3	n.o.	23 ± 2*	n.o.

The phospholipid composition was determined as described in 'Materials and methods'. ^{31}P-NMR data for the isolated NADPH-cytochrome reductase are not included because they could not be accurately quantified. The results represent the mean ± standard error of the mean of seven experiments with different reductase and microsomal preparations. The literature values for the phospholipid composition of rat liver microsomes of untreated Wistar rats are taken from Davison and Wills [32].
n.o. = not observed

‡ For thin layer chromatography analysis the amount of PS and PI is presented as the sum of both phospholipids as they gave rise to a single spot on the thin layer chromatography plates.

* significantly different from the value in the microsomes at $P<0.02$

Together, the data in Table 5.2 demonstrate that the phospholipid composition of the microsomal membrane as determined in the present study by chemical analysis and ^{31}P-NMR corresponds well to values previously reported in the literature [32]. Furthermore, the data show that,

compared to the microsomal membrane, the isolated NADPH-cytochrome reductase is enriched in PS / PI, whereas the relative amounts of PC and PE are comparable. This result corresponds to the results from the ^{31}P-NMR experiments (Figure 5.1) demonstrating a relative enrichement of PS (0.48 ppm) and PI (0.16 ppm) compared to PC (0.02 ppm) and PE (0.61 ppm).

Altogether it is concluded that NADPH-cytochrome reductase appears to bind preferentially the negatively charged phospholipids PS and PI.

5.4.3 The effect of the purification procedure on the phospholipid composition of isolated NADPH-cytochrome reductase preparations

In the experiments described above, the phospholipid composition of purified NADPH-cytochrome reductase was demonstrated to be relatively enriched in PS and PI. However, during the purification procedure, in theory, one (or more) of the isolation steps may remove selectively certain phospholipids from the protein resulting in a different phospholipid composition of the isolated protein compared with that of the microsomal membrane. Especially the method for membrane solubilisation might influence the phospholipid content of the purified membrane protein [33]. Therefore, it was investigated whether the solubilisation procedure affects the phospholipid composition of the solubilised NADPH-cytochrome reductase .

Liver microsomes from phenobarbital pretreated rats were solubilised with different mixtures of various detergents after which the phospholipid compositions of the solubilised proteins were determined by ^{31}P-NMR. The detergents used were sodium cholate and sodium deoxycholate, both ionic detergents, and Emulgen 911 and Lubrol PX, both non-ionic detergents. Solubilisation of liver microsomes with 0.63% sodium cholate, 0.63% sodium deoxycholate, 0.63% Lubrol, 0.63% Emulgen, 0.31% sodium cholate / 0.31% Lubrol, 0.31% sodium cholate / 0.31% Emulgen, 0.31% sodium deoxycholate / 0.31% Lubrol, 0.31% sodium deoxycholate / 0.31% Emulgen or 0.37% sodium deoxycholate / 0.71% Lubrol (used in the isolation procedure of NADPH-cytochrome reductase) resulted in ^{31}P-NMR spectra similar to the one presented in Figure 5.2 for the solubilisation with 1.11% sodium cholate / 0.55% Lubrol (used in the isolation procedure of cytochrome P450).

Comparison of the ^{31}P-NMR spectrum of the solubilised protein preparation (Figure 5.2) with the ^{31}P-NMR spectra of the purified NADPH-cytochrome reductase and the microsomal membrane (Figure 5.1[a] and [b]) demonstrates that the spectrum of the solubilised preparation resembles that of the isolated NADPH-cytochrome reductase and is different from the microsomal membrane. These results indicate that, irrespective of the concentration and type of detergent used for the solubilisation, the phospholipid composition of the solubilised microsomal protein preparation is different of that of the membrane.

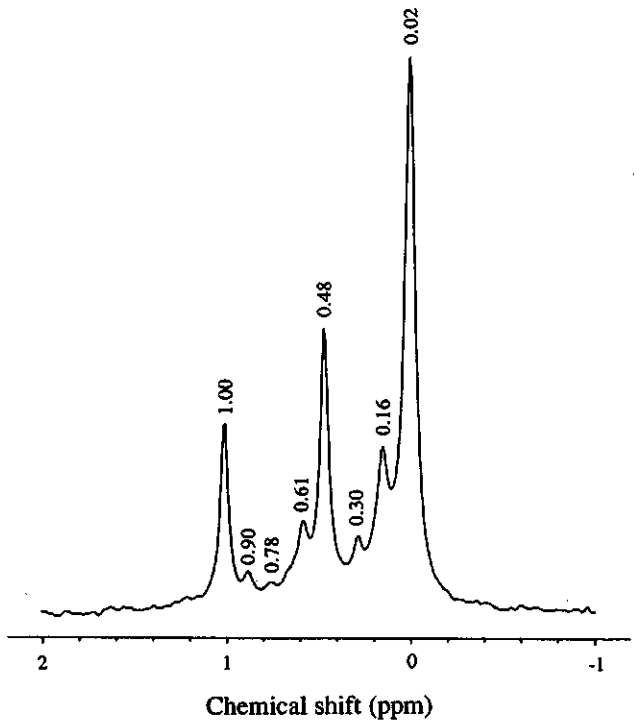

Figure 5.2: ^{31}P-NMR spectrum of a solubilised microsomal protein preparation. Solubilisation was carried out as described in 'Materials and methods' with 1.11% sodium cholate and 0.55% Lubrol. The protein preparation was prepared for ^{31}P-NMR as described in 'Materials and methods'.

To investigate whether differences in the phospholipid contents for the purified NADPH-cytochrome reductase and the microsomal membrane is mainly determined by the solubilisation procedure, the phospholipid contents of the protein preparations obtained during the affinity column chromatography of the NADPH-cytochrome reductase purification were compared. During the last step of the isolation NADPH-cytochrome reductase is separated from the remaining proteins by a 2'5'ADP-sepharose column. The ^{31}P-NMR spectrum of a concentrated sample containing these collected protein impurities is presented in Figure 5.3 and can be compared to the ^{31}P-NMR spectrum of the purified NADPH-cytochrome reductase originating from the same isolation procedure (Figure 5.1[a]).

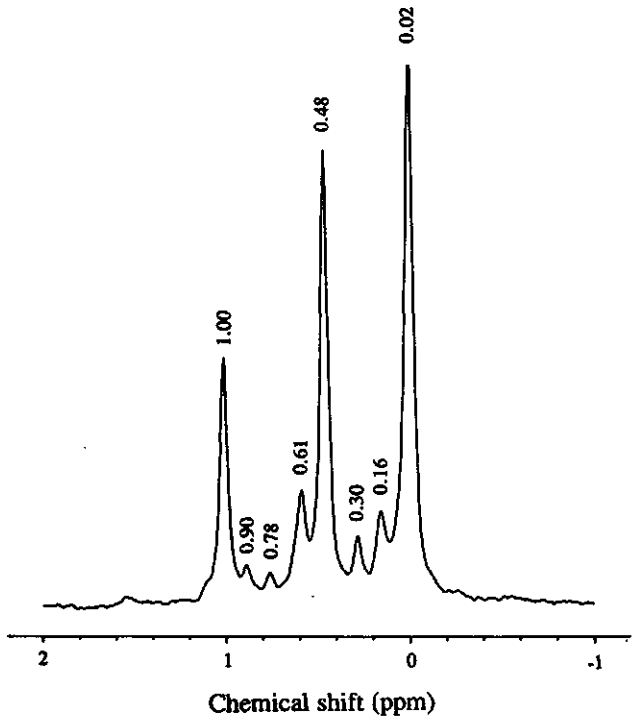

Figure 5.3: ^{31}P-NMR spectrum of the protein fraction which did not bind to the 2'5'ADP-sepharose column used for the NADPH-cytochrome reductase purification.

The protein preparation was prepared for ^{31}P-NMR as described in 'Materials and methods'.

Comparison of the ^{31}P-NMR spectra of NADPH-cytochrome reductase and the 2'5'ADP-sepharose effluent shows that the phospholipid composition of the protein preparation which does not bind to the 2'5'ADP-sepharose column, is different from the phospholipid composition of the isolated NADPH-cytochrome reductase. In particular, the increase in the peak at 0.48 ppm (PS) was even more pronounced in the effluent protein fraction than already observed in the isolated NADPH-cytochrome reductase, whereas the relative increase in the peak at 0.16 ppm (PI) was only observed for the NADPH-cytochrome reductase samples. Together, these results demonstrate that the phospholipid composition of the isolated NADPH-cytochrome reductase preparation is not solely determined by its solubilisation from the membrane, but it also reflects a special affinity of the protein for the different phospholipids.

5.4.4 The effect of the phospholipid headgroup on the DPH-PC dependent quenching of tryptophan fluorescence of NADPH-cytochrome reductase

The experiments described above point to a specific relationship between NADPH-cytochrome reductase and PS and PI. In additional experiments we further investigated a possible special interaction of NADPH-cytochrome reductase with PS, PI and other phospholipids by studying the effect of PC, PE, PS and PI on the DPH-PC mediated quenching of the tryptophan fluorescence of NADPH-cytochrome reductase. Figures 5.4 and 5.5 present the results obtained. From these data it follows that upon the addition of DPH-PC to a solution of NADPH-cytochrome reductase its tryptophan fluorescence at λ_{em} = 340 nm decreases. The decrease in the NADPH-cytochrome reductase tryptophan fluorescence is accompanied by an increase in the fluorescence emission spectrum of DPH-PC (400-500 nm). Furthermore, Figure 5.4 demonstrates that the apparent decrease in the fluorescence emission of tryptophan residues at 340 nm does not result from an inner filter effect by DPH-PC as DPH-PC does not demonstrate its characteristic emission spectrum at λ_{ex} = 280 nm (inset a, Figure 5.4). More likely, the appearance of the fluorescence emission spectrum of DPH-PC is explained by the energy transfer from tryptophan residue(s) of NADPH-cytochrome reductase to DPH-PC molecules located in the direct vicinity of these tryptophans. Because energy transfer is effective over distances ranging to 50 Å [34], the DPH-

PC molecules must be located within this distance from the tryptophan residues. The excitation of these DPH-PC molecules results in the typical DPH-PC fluorescence emission spectrum between 400 and 500 nm (inset b, Figure 5.4).

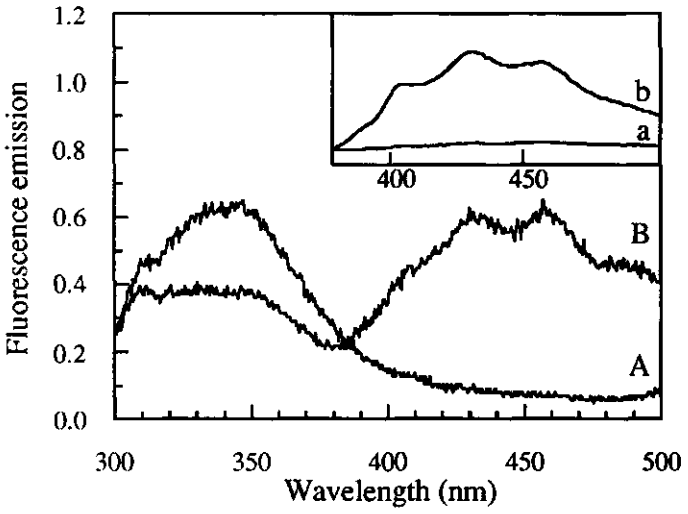

Figure 5.4: Fluorescence emission spectrum of 75 pmol NADPH-cytochrome reductase in the presence of 20 nmol PC (A) or 20 nmol PC / DPH-PC (1:1) (B).

The DPH-PC mediated quenching of tryptophan fluorescence of NADPH-cytochrome reductase was determined as described in 'Materials and methods'. Inset: the fluorescence emission spectrum of 20 nmoles of DPH-PC in 0.1 M potassium phosphate buffer (pH = 7.5) at $\lambda_{excitation}$ = 280 nm (a) and $\lambda_{excitation}$ = 355 nm (b).

Figure 5.5 presents the results from experiments in which the tryptophan fluorescence was measured as a function of the molar fraction of DPH-PC, mixed with other phospholipids (PC, PE, PS or PI). The results obtained demonstrate that - as expected - increasing the molar fraction of DPH-PC results in a gradual decrease in the tryptophan fluorescence. However, even a 150 times molar excess of DPH-PC over NADPH-cytochrome reductase cannot completely quench the tryptophan fluorescence of the protein. This means that either some of the tryptophans are deeply burried in the reductase molecule and their

fluorescence cannnot be quenched by DPH-PC or a finite distance exists between DPH-PC and the tryptophan residues.

Furthermore, Figure 5.5 shows that at a molar DPH-PC fraction of about 0.5 in the PS- and PI-system the tryptophan fluorescence is lower than the tryptophan fluorescence at a molar DPH-PC fraction of 1. Since this phenomenon was only observed in the PS- and PI-system it could be related to the negative charge of these phospholipids and may be connected with either a charge dependent conformational change in the reductase structure or a different kind of association of DPH-PC with reductase in the presence of PS and PI.

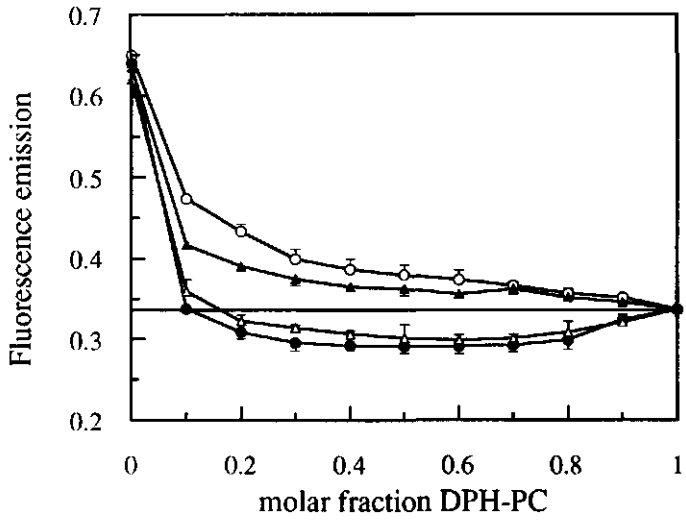

Figure 5.5: The effect of PC (o-o), PE (▲-▲), PS (●-●) and PI (Δ-Δ) on the DPH-PC dependent quenching of the tryptophan fluorescence of NADPH-cytochrome reductase.

The DPH-PC mediated quenching of tryptophan fluorescence of 75 pmol NADPH-cytochrome reductase ml^{-1} was determined as described in 'Materials and methods'. The results present the mean ± standard error of the mean of three experiments.

5.4.5 The effect of the phospholipid headgroup on the kinetics of the cytochrome P450 IIB1 dependent O-dealkylation of pentoxyresorufin

In addition to the effect of phospholipids with different headgroups on the DPH-PC dependent quenching of the tryptophan fluorescence of NADPH-cytochrome reductase, their effect on the kinetics of cytochrome P450 IIB1-dependent O-dealkylation of pentoxyresorufin was

investigated. This was done to characterise a possible special role for PS and PI in the NADPH-cytochrome reductase / cytochrome P450 system. The results from these experiments are presented in Table 5.3.The results demonstrate that compared to a system without added phospholipids, the addition of phospholipids to the reconstituted system results in a significant increase in V_{max} whereas the substrate apparent K_m is not affected. However, when the kinetic parameters of the pentoxyresorufin O-dealkylation in the presence of phospholipid (mixtures) are compared to one another, the data demonstrate that the presence of PC and a 1:1 (w/w) mixture of PC with PE affect the substrate's apparent K_m and V_{max} in a similar way. On the other hand, the use of a phospholipid mixture of PC and the negatively charged PI results in a significantly smaller increase in the apparent V_{max}. An opposite effect on V_{max} was observed when cytochrome P450 and NADPH-cytochrome reductase were preincubated with a mixture of PC and PS. Addition of the negatively charged PS resulted in a significant larger increase in the apparent V_{max} compared to PC and PC / PE. These results imply that the V_{max} of pentoxyresorufin-O-dealkylation in the presence of PC / PS is significantly higher than the V in the presence of PC / PI.

Table 5.3: The effect of (mixtures of) phospholipids with different headgroups on the kinetics of the pentoxyresorufin-O-dealkylation by reconstituted cytochrome P450 IIB1.

phospholipid(s)	K_m (μM)	V_{max} (nmol.min^{-1}.nmol P450^{-1})
none	0.65 ± 0.07	0.12 ± 0.01**
PC	0.77 ± 0.02	0.30 ± 0.01
PC / PE (1:1 $^{w}/_{w}$)	0.72 ± 0.02	0.31 ± 0.02
PC / PS (1:1 $^{w}/_{w}$)	0.72 ± 0.01	0.37 ± 0.01*
PC / PI (1:1 $^{w}/_{w}$)	0.72 ± 0.01	0.22 ± 0.02*

The O-dealkylation of pentoxyresorufin by cytochrome P450 IIB1 was determined as described in 'Materials and methods'. The results represent the mean ± standard error of the mean of three experiments with two different protein preparations. Substrate apparent K_m and V_{max} values were determined by fitting the experimental data to the Michaelis-Menten equation.

* significantly different from the value for PC at P<0.05.

** significantly different from the values with phospholipids at P<0.005.

5.5 Discussion

In the present study the interaction of NADPH-cytochrome reductase with phospholipids was investigated using ^{31}P-NMR, thin layer chromatography combined with chemical analysis, fluorescence spectroscopy and kinetic experiments in a reconstituted cytochrome P450 IIB1 system. The results from the ^{31}P-NMR experiments demonstrate a significant difference between the phospholipid composition of the microsomal membrane and the composition of the phospholipids that remain associated to NADPH-cytochrome reductase upon purification of the protein. The ^{31}P-NMR data suggest a relative enrichment of the purified NADPH-cytochrome reductase in PS or LPC (0.48 ppm) and PI (0.16 ppm). Unequivocal identification and quantification of the phospholipid contents of the various samples came from thin layer chromatography analysis combined with chemical analysis of extracted spots. The results from these experiments were in agreement with those from the NMR study and demonstrated that the peak at 0.48 ppm results from PS and thus, the isolated NADPH-cytochrome reductase preparations were enriched in PS and PI compared to the microsomal membrane.

The enrichement of NADPH-cytochrome reductase in PS and PI, observed in the present study, is not in line with the results reported by Narayanasami *et al.* [30] who observed that in *E.coli* -expressed forms of NADPH-cytochrome reductase the major form of phospholipid that remains bound to reductase upon its purification, is PE. However, the fact that in their study the protein was expressed in *E.coli.* might also be the reason for this differential observation because in *E. coli* PE is the most abundant form of phospholipid [35].

Additional data demonstrate that the PI / PS enrichment which occurs upon solubilisation of the proteins from the membrane is independent of the type or concentration of the detergent used for solubilisation of the microsomal proteins and to be to some extent dependent on the nature of the solubilised protein.

Additional results show that (i) PS and PI affect the DPH-PC dependent quenching of the tryptophan fluorescence of NADPH-cytochrome reductase in a different way than PC and PE and (ii) preincubation of cytochrome P450 IIB1 and NADPH-cytochrome reductase with a mixture of PC with either PS or PI results in a

significantly different effect on the apparent V_{max} of the pentoxyresorufin-O-dealkylation with respect to preincubation of the proteins with either a mixture of PC and PE or PC alone. These observations show an PS and PI dependent change in the NADPH-cytochrome reductase conformation, resulting on the one hand in an improved interaction with DPH-PC and a concomitant increase in the quenching of the reductase's tryptophan fluorescence, due to enhanced energy transfer to DPH-PC, and on the other hand in a possible change in its interaction with cytochrome P450 resulting in an effect on V_{max}. The increase in V_{max} by PS might, for example, be achieved by improving the alignment of the proteins through charge-charge interactions of protein(s) and phospholipid or through neutralisation of repelling positive charges on the surface of NADPH-cytochrome reductase and cytochrome P450 IIB1. This improved interaction between the proteins may result in an increased rate of electron transfer and thus an increased apparent V_{max}. On the other hand, the different effects of PI and PS on the apparent V_{max} might also result from a different effect of the two phospholipids on other, not yet characterized properties of the proteins of the reconstituted system. The fact that the also negatively charged PI affects the kinetic parameters in a different manner than PS - PI causing a decrease in the apparent V_{max} - indicates that the stimulating effect of PS on the pentoxyresorufin-O-dealkylating activity of the reconstituted system must at least in part be due to other factors than its negative charge.

At this stage it should be stressed that the results of the kinetic experiments were obtained with an *in vitro* system which differs significantly from the *in vivo* , membrane bound situation. In the reconstituted system of the present study the proteins are not incorporated in a membrane and the molar cytochrome P450 : reductase ratio is much lower than the one known to exist in the endoplasmatic reticulum membrane (1:1 versus 30-15:1). This makes extrapolation of the results from the *in vitro* system to the situation in the endoplasmatic reticulum membrane to some extent speculative although the results do provide a basis for formulation of new working hypotheses.

The importance of the negatively charged phospholipids in the cytochrome P450 enzyme system has also been reported by some other investigators. Ingelman-Sundberg *et al.* [11] reported an apparent linear relationship between the negative charge of reconstituted vesicles and the

rate of cytochrome P450 LM_2 (= IIB1 from rabbit liver) catalysed O-dealkylation of p-nitroanisole and 7-ethoxycoumarin. When incorporated into vesicles composed of PE and PS the ethoxycoumarin-O-dealkylating activity of cytochrome P450 LM_2 was about double that of systems in which the proteins are incorporated into vesicles composed of PE or PC alone. This result is in line with the increased V_{max} for P450 IIB1 catalysed pentoxyresorufin-O-dealkylation reported in the present study. In the previous study however, only PS was used to increase the negative charge of the phospholipid vesicle; studies on the effect of PI on the ethoxycoumarin-O-dealkylating activity were not reported. In another study, Blanck *et al.* [36] reported that the dissociation constant for the catalytically active P450 LM_2 / reductase complex in the presence of a 3:1 mixture of PE and PS is comparable to the dissociation constant in the presence of a microsomal lipid extract and 10 times smaller than in the presence of PC. This observation is also in line with the higher V_{max} for the cytochrome P450 IIB1 catalysed pentoxyresorufin-O-dealkylating activity in the presence of PS reported in this study. However, in the presence of the other naturally abundant negatively charged phospholipid, namely PI, V_{max} of the pentoxyresorufin-O-dealkylation by the reconstituted cytochrome P450 IIB1 system was decreased. Thus, the results of the present study not only demonstrate a preference of NADPH-cytochrome reductase for the negatively charged PS and PI, but also point at possibilities for regulation of the kinetics of a reconstituted cytochrome P450 IIB1 system by PS (increasing V_{max}) and PI (decreasing V_{max}). Furthermore, the results clearly demonstrate that in addition to the cytochrome P450 component, the NADPH-cytochrome reductase component of the system might add to the phospholipid preference of the enzyme system for negatively charged phospholipids.

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Chapter 6

Experimental and theoretical study on the redoxcycling of resorufin by solubilized and membrane-bound NADPH-cytochrome reductase

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6.1 Summary

The present study describes both experimental and theoretical data on the redox cycling of resorufins catalyzed by NADPH-cytochrome reductase. At 1-5 μM concentrations at physiological pH, the redox cycling of ethoxy- and pentoxyresorufin was shown to be far more efficient than the redox cycling of their product from the cytochrome-P450 dependent O-dealkylation, resorufin (7-hydroxyphenoxazone). This was shown to result from the fact that i) the protonated form of the resorufin is a much better substrate for redox cycling than the deprotonated resorufin O-anion and that ii) at physiological pH the redox cycling active protonated form is present at only 1-4 % of the total amount of resorufin.

In addition to experimental data, AM1 molecular orbital computer calculations provided evidence for the difference in redox cycling capacity between the resorufin O-anion and its protonated form. The energy of the lowest unoccupied molecular orbital (E_{LUMO}) of the resorufin O-anion is higher than the E_{LUMO} value for the protonated form. This low E_{LUMO} value of the protonated form can be taken as a parameter for its easier reduction. Furthermore, computer calculations demonstrated one electron reduction of the protonated form to be energetically favourable by 363.5 kJ/mol, compared to one electron reduction of the deprotonated O-anionic form.

Additional AM1 molecular orbital computer calculations indicated that the one-electron reduced resorufin will become protonated at the O-atom of the intramolecular semiquinoneimine moiety before reduction by a second electron becomes likely. Finally, redox cycling of resorufin by solubilized and membrane incorporated NADPH-cytochrome reductase provided evidence that membrane surroundings increase the concentration of the protonated form of resorufin. This effect is achieved either by favored partitioning of the protonated resorufin into the membrane and / or by an effect of the membrane on the protonation equilibrium of resorufin in favour of the protonated form.

6.2 Introduction

The 7-alkoxyresorufins are frequently used in biochemical toxicology to study the activity of cytochromes P450 in liver microsomal preparations.

Cytochromes P450 catalyze the O-dealkylation of these substrates to the highly fluorescent resorufin (7-hydroxyphenoxazone) [1-3]. Recently, it was reported that this series of alkoxyresorufins and resorufin, containing an intramolecular quinoneimine function, are substrates for redox cycling, catalyzed by NADPH-cytochrome reductase (E.C.1.6.2.4.) [4]. During redox cycling electrons from the NADPH-cytochrome reductase are not passed to cytochrome P450 for substrate monooxygenation, but are passed to the quinone(imine), resulting in semiquinone(imine)s and/or dihydroquinone(imine)s. As these one or two electron reduced forms pass their electrons on to molecular oxygen they give rise to the formation of toxic, reactive oxygen species like superoxide anions and hydrogen peroxide, from which, in a Fenton-type reaction, hydroxyl radicals can be formed [5]. This reoxidation of the semi- or dihydroquinone(imine)s by oxygen results in their conversion to the original quinone(imine)s which can participate in a new redox cycle.

In the present paper the redox cycling of the resorufin derivatives is investigated in more detail. At physiological pH and 1-5 μM concentration, the alkoxyresorufins appear to be better substrates for NADPH-cytochrome reductase dependent redox cycling than their O-dealkylation product resorufin. Furthermore, membrane incorporated NADPH-cytochrome reductase is shown to catalize higher redox cycling rates than solubilized NADPH-cytochrome reductase at a given concentration of resorufin.

Experiments and computer AM1 molecular orbital calculations presented in this paper demonstrate the reason underlying these effects.

6.3 Materials and methods

6.3.1 Purification of NADPH-cytochrome reductase

NADPH-cytochrome reductase was isolated from liver microsomes of phenobarbital-treated female Wistar rats essentially as described by Yasukochi and Masters [6]. Lubrol PX (Sigma, St. Louis, MO, USA) was used instead of Renex 690 and all buffers contained 100 mM phenylmethanesulfonyl fluoride (Merck, Darmstadt, FRG) to inhibit protease activies. Excess detergent and phospholipid was removed by additional washing of the 2'5'-ADP-Sepharose column bound protein using the prescribed buffers without the detergents lubrol and

deoxycholate (Merck, Darmstadt, FRG). The enzyme was eluted using 20 mM potassium phosphate buffer (pH 7.7) containing 10 mM adenosine 2'(3')-monophosphate (Sigma, St. Louis, MO, USA), 20 % (v/v) glycerol, 0.1 mM EDTA, 0.1 mM dithiotreitol (Boehringer, Mannheim, FRG), 0.1 mM phenylmethanesulfonyl fluoride and 0.2 % (w/v) sodium deoxycholate. The final preparation was concentrated over an Amicon YM-30 filter and dialysed 4 times against 1 liter 20 mM potassium phosphate buffer (pH 7.7) containing 20 % (v/v) glycerol and 0.1 mM EDTA to remove the deoxycholate and adenosine 2'(3')-monophosphate.

NADPH-cytochrome reductase activity was measured as described by Philips and Langdon [7] using cytochrome *c* as the final electron acceptor. One µmol cytochrome *c* reducing activity was taken as the unit to quantify the NADPH-cytochrome reductase activity. Protein was measured according to Lowry *et al.* [8] using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard.

The final detergent-free preparation had a specific activity of at least 35 units/mg protein, was homogeneous as judged by SDS/PAGE, carried out as described by Laemmli [9] and stored in small portions at -20 °C until use.

6.3.2 Reconstituted NADPH-cytochrome reductase systems

Two types of systems with isolated NADPH-cytochrome reductase were used. The first one is referred to as solubilized NADPH-cytochrome reductase. For this system the isolated reductase preparation is used as such, if necessary diluted in 0.1 M potassium phosphate buffer (pH 7.5) containing 20% (v/v) glycerol just before use.

In addition, membrane incorporated NADPH-cytochrome reductase was used. Membrane incorporation of the isolated enzyme was achieved by the so called cholate dialysis method, carried out essentially as described by Taniguchi *et al.* [10]. In short 6.25 units of the isolated NADPH-cytochrome reductase were mixed with 0.15 mg L-α-dilauroyl-*sn*-glycero-3-phosphocholine (PC di-12:0) (Sigma, St. Louis, MO, USA) and diluted with at least two volumes of 50 mM potassium phosphate buffer (pH 7.25) containing 0.1 mM EDTA, 0.1 mM dithiothreitol and 1 % (w/v) sodium cholate (Merck, Darmstadt, FRG). Two ml of this mixture were dialysed for 48 hours against 2 times 2 liters of 50 mM potassium phosphate buffer (pH 7.25) containing 0.1 mM EDTA and 0.1 mM dithiotreitol.

6.3.3 Redoxcycling of resorufins

The redox cycling of resorufins was measured in 0.1 M potassium phosphate buffer (pH 7.5) at room temperature. Both the solubilized and the membrane incorporated NADPH-cytochrome reductase preparation were used in such dilutions that 10 µl reductase preparation added per ml reaction mixture resulted in a final concentration of 0.025 units reductase/ml. The desired concentrations of resorufin (Janssen Chimica, Beerse, Belgium), ethoxy- or pentoxyresorufin (both from Boehringer, Mannheim, FRG) were added as 1 % (v/v) of a 100 times concentrated solution in dimethylsulfoxide. Reactions were started by addition of NADPH (0.15 mM final concentration) and followed by the decrease in the NADPH absorption at 340 nm. Reactions were corrected for resorufin independent NADPH oxidation which was routinely less than 5% of the resorufin dependent one.

The amount of H_2O_2 was determined indirectly by production of formaldehyde during oxidation of methanol essentially as described by Werringloer [11] using the method of Nash [12]. Oxygen consumption was measured using the Clark oxygen electrode at 25 °C.

6.3.4 Molecular orbital computer calculations

Computer calculations were performed on a Silicon Graphics Iris 4D/85 with Quanta/Charmm (Polygen Ltd., Reading, U.K.) using the semi-empirical molecular orbital AM1 method from the AMPAC program obtained from the Quantum Chemistry Program Exchange (QCPE program no. 506(2.1)) (Indiana University, Bloomington, IN, USA). Geometries were optimized for all bond lengths, bond angles and torsion angles. Geometry was optimized using the Fletcher-Powell criteria. For all calculations Herberts test was satisfied in Fletcher-Powell and the self-consistent field was achieved.

The results from these computer calculations represent the data for the compounds in vacuo, a situation different from the one in biological systems, where solvation and a different polarity of the direct environment (dielectric constant) might influence Coulomb and Frontier orbital characteristics of the substrates to the in-vacuo situation. In the present study, however, a series of related resorufins is compared and it is assumed that deviations from the in-vacuo situation will be similar for all

substrates, and will not influence relative differences between the various resorufins.

6.3.5 Statistical analysis of data

Data are presented as the mean ± standard error of the mean (SEM) and statistical analysis was carried out using Student's t-test, for unpaired samples. The null-hypothesis was rejected at $P \leq 0.05$.

6.4 Results

6.4.1 Redox cycling of resorufin and its 7-alkoxy derivatives

The redox cycling of resorufin and two of its 7-alkoxy derivatives by isolated, solubilized NADPH-cytochrome reductase was investigated. From Table 6.1 it can be seen that the observed reaction for the resorufin derivatives can indeed be ascribed to redox cycling. For the different substrates the NADPH oxidation is accompanied by proportional oxygen

Table 6.1: NADPH oxidation, H_2O_2 production and O_2 consumption during redox cycling of resorufin and pentoxyresorufin by solubilized and membrane incorporated NADPH-cytochrome reductase.

reconstituted system	Time (min.)	NADPH oxidation ($nmol.ml^{-1}$)	H_2O_2 production ($nmol.ml^{-1}$)	O_2 consumption ($nmol.ml^{-1}$)
solubilized reductase + resorufin	5 min.	16	13	14
	10 min.	30	26	29
	15 min.	43	38	42
solubilized reductase + pentoxyresorufin	5 min.	20	17	15
	10 min.	39	33	30
	15 min.	58	46	44
membrane incorporated reductase + resorufin	5 min.	30	24	35
	10 min.	58	48	58
	15 min.	82	69	71

NADPH oxidation, H_2O_2 production and O_2 consumption were measured in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 units/ml reductase and 50 µM resorufin or 5 µM pentoxyresorufin essentially as described in 'Materials and methods'.

Table 6.2: Kinetic data from Lineweaver-Burk plots for the NADPH-cytochrome reductase dependent redox cycling of resorufin and its 7-alkoxy derivatives at pH 7.5.

substrate	apparent K_m (μM)	V_{max} (nmol NADPH oxidized. min^{-1}. unit reductase^{-1})
resorufin	125 ± 11	308 ± 29
ethoxyresorufin	4 ± 1	336 ± 26
pentoxyresorufin	4 ± 1	282 ± 77

Solubilized NADPH-cytochrome reductase was used. The values represent the mean ± SEM of four experiments.

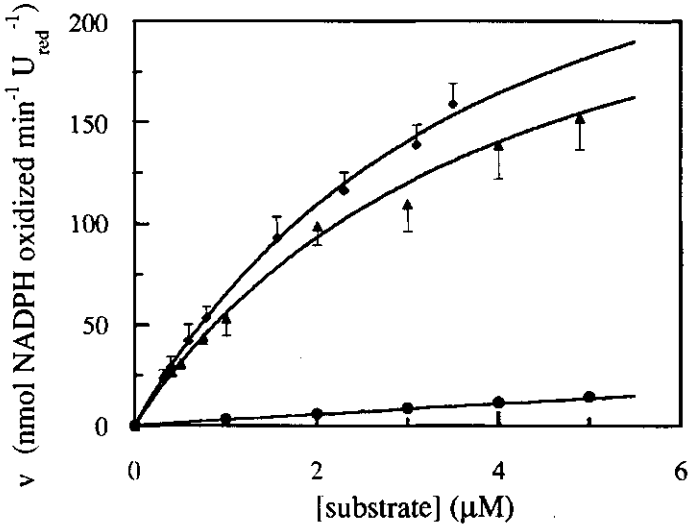


Figure 6.1: Redox cycling of resorufin (●-●), ethoxyresorufin (♦-♦) and pentoxyresorufin (▲-▲) by solubilized NADPH cytochrome reductase at pH 7.5.

Redoxcycling was measured as described in 'Materials and methods', by NADPH oxidation at 340 nm. The values represent the mean ± SEM of three experiments.

consumption and H_2O_2 formation. When the redox cycling of the three substrates was investigated as a function of their concentration, ethoxy- and pentoxyresorufin appeared to be far more efficient than resorufin (Figure 6.1). Data from kinetic studies, presented in Table 6.2, show that

this is mainly caused by the about thirty times larger apparent K_m for resorufin. This results in a low rate of redox cycling of resorufin at the 1-5 μM concentrations used in the experiment of Figure 6.1. Increasing the concentration results in a V_{max} that is comparable to those observed for ethoxy- and pentoxyresorufin (Table 6.2).

6.4.2 Effect of pH on resorufin redox cycling

It is demonstrated in Figure 6.2 that the redox cycling of resorufin is increased upon lowering the pH in spite of an inactivation of the NADPH-cytochrome reductase (measured by cytochrome *c* reduction). In contrast the redox cycling of the two alkoxyresorufins decreases to the same extent as the cytochrome *c* reducing activity of the enzyme. Results from kinetic studies on the redox cycling of resorufin at pH values below 7.5, namely 7.0 and 6.5, showed that a decrease in the pH is accompanied by a decrease in the K_m of NADPH-cytochrome reductase for resorufin (Table 6.3). The V_{max} appeared to be not significantly affected by changes in the pH.

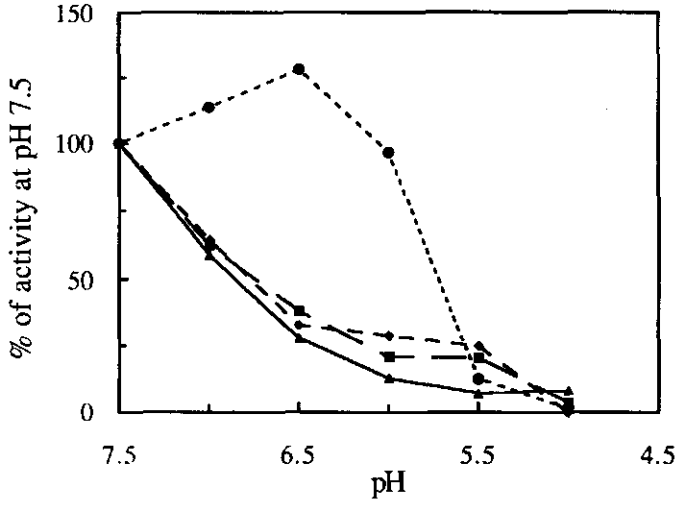


Figure 6.2: Redox cycling of resorufin (●-●), ethoxyresorufin (◆-◆) and pentoxyresorufin (▲-▲), and cytochrome c reduction (■-■) by solubilized NADPH-cytochrome reductase as a function of decreasing pH.

Assays were carried out as described in 'Materials and methods'. The pH was varied by varying the pH of the potassium phosphate buffer.

Table 6.3: Kinetic data from Lineweaver-Burk plots for the NADPH-cytochrome reductase dependent redox cycling of resorufin at different pH.

pH	apparent K_m (μM)	V_{max} (nmol NADPH oxidized .min^{-1} .unit $reductase^{-1}$)
7.5	125 ± 11	308 ± 29
7.0	53 ± 4**	281 ± 25
6.5	33 ± 6*,***	264 ± 9

Solubilized NADPH-cytochrome reductase was used. The values represent the mean ± SEM of three experiments. Redox cycling was measured as described in 'Materials and methods'.

* = significantly different from the value at pH 7.0 for P<0.05

** = significantly different from the value at pH 7.5 for P<0.005

*** = significantly different from the value at pH 7.5 for P<0.001

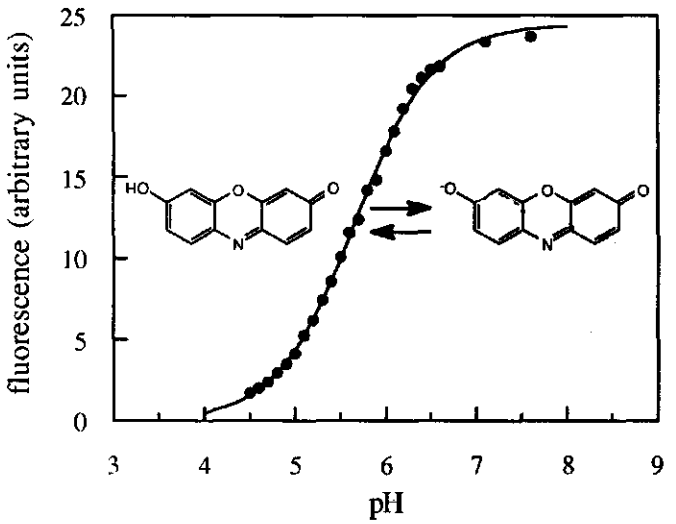


Figure 6.3: Determination of the pK_a of resorufin by fluorescence spectroscopy. At λ_{ex} = 530 nm, the intensity of the fluorescence emission at λ_{em} = 585 nm was determined for resorufin dissolved in 0.1 M potassium phosphate buffer of varying pH. For reasons indicated in the text, the fluorescence is ascribed to the deprotonated resorufin O-anion.

Alltogether, these data suggest that a protonated and deprotonated form of resorufin show different capacities for redox cycling, the protonated one being more active.

From the results presented in Figure 6.3 the pK_a for resorufin was calculated to be 5.7. This means that an increase in the ratio protonated / deprotonated resorufin will occur with decreasing pH in the range where the redox cycling of resorufin is increased.

Based on the highly coloured and fluorescent character of resorufin at pH values higher than its pK_a, and on the observation that at pH values below the pK_a the colour of the resorufin changes from pink to orange, thereby becoming similar to that of its alkylated derivatives ethoxy- and pentoxyresorufin, it is concluded that the acid-base equilibrium between the protonated resorufin and its deprotonated O-anion is involved, as indicated in Figure 6.3.

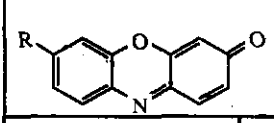
From the pK_a value of 5.7 it can be calculated that going from pH 7.5 to 6.5, the relative amount of the protonated resorufin increases from 1.6 % to 13.7 % of the total amount of resorufin added. If the protonated form is indeed the only form participating in the redox cycling this explains the increase in resorufin dependent redox cycling activity with decreasing pH (Figure 6.2), and the decrease in the apparent K_m with decreasing pH (Table 6.3).

6.4.3 AM1 molecular orbital calculations.

As the results presented above suggested that protonated resorufin would be a much better substrate for redox cycling than its deprotonated, O-anionic form, computer calculations were carried out to see whether the phenomenon can be ascribed to molecular characteristics of this anionic form. Table 6.4 shows the results of AM1 computer calculations. From these data it can be derived that the energy of the lowest unoccupied molecular orbital (E_{LUMO}), the molecular orbital into which the electron will be placed upon the one electron reduction, might indeed provide a parameter to predict whether compounds are likely candidates for redox cycling or not. The E_{LUMO} of the resorufin O-anion is significantly higher than the E_{LUMO} for the protonated resorufin and the E_{LUMO} for the alkoxyresorufins. This indicates that one electron reduction of the resorufin anion will require more energy than one electron reduction of either the protonated resorufin or the resorufin ethers. This is confirmed by the difference in the final heats of formation between the one electron reduced form and the oxidized form (ΔHF) calculated for the different resorufins (Table 6.4). From these data it can be derived that one electron

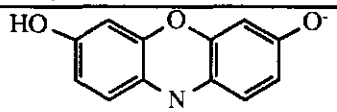
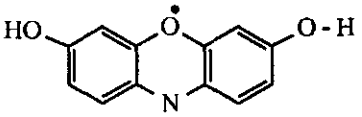
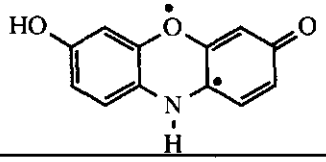
reduction of the protonated resorufin form is 363.5 kJ/mol energetically favourable over one electron reduction of the resorufin O-anion.

Table 6.4: Calculated E_{LUMO} values of the resorufin derivatives and difference in final heats of formation for the one electron reduced forms.

resorufin derivative 	R=	E_{LUMO} (eV)	ΔHF (kJ/mol)
resorufin	-OH	-1.71	-219.2
resorufin anion	$-O^-$	+2.06	+144.3
ethoxyresorufin	$-OC_2H_5$	-1.65	-215.1
pentoxyresorufin	$-OC_5H_{11}$	-1.63	-214.2

Calculations were carried out as described in 'Materials and methods'. The heat of formation difference between the one electron reduced form and the oxidized form of the resorufins (ΔHF) is calculated as $HF_{1e^-red} - HF_{ox}$.

Table 6.5: Calculated energies of the single occupied molecular orbital (SOMO) in which the electron has to be placed upon one electron reduction of the resorufin semiquinoneimine radical, and final heats of formation (HF) for the O- or N-protonated one electron reduced resorufin forms.

molecular structure	E_{SOMO} (eV)	HF (kJ/mol)
	-0.28	
	-4.58	-106.7
	-4.91	-91.6

Calculations were carried out as described in 'Materials and methods'. In the molecular structures depicted, the charge and the unpaired electron were situated on the atom with, respectively, the highest negative charge and the highest spin density. The actual, calculated charge and spin distributions are depicted in Figure 6.4.

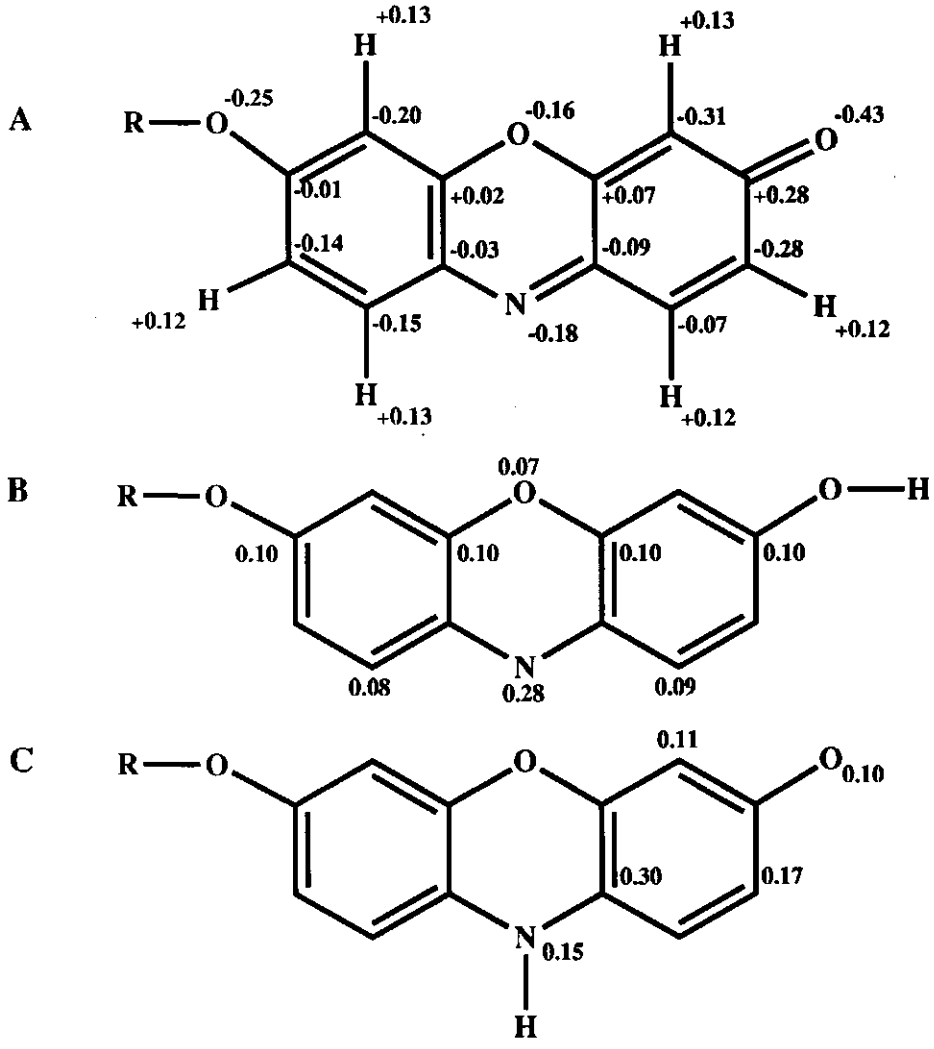


Figure 6.4: Calculated charge distribution for the one electron reduced resorufin (a) and calculated spin distribution for the O-protonated (b) and N-protonated (c) form of one electron reduced resorufin.

Values given represent the net atomic charges (a) or the total spin density (b+c) on the atoms. For the spin distribution calculation only the spin densities larger than 0.03 are presented. Calculations were carried out as described in 'Materials and methods'.

In Table 6.5 similar AM1 calculation results for non-protonated and O- or N-protonated one electron reduced resorufin are presented. In analogy to the arguments refered to above, it can be concluded from these results

that the semiquinoneimine form has to be protonated before it might become reduced in a second redox cycling step. It is proposed by Dutton and Parkinson [4] that this protonation takes place at the nitrogen atom of the intramolecular semiquinoneimine moiety. However, the charge distribution calculated for the one electron reduced resorufin (Figure 6.4[a]) suggests that protonation of the oxygen of the semiquinoneimine moiety is more likely than protonation of the nitrogen atom. This follows from the observation that the negative charge, located on the O-atom of the intramolecular semiquinoneimine moiety, is much larger than the one located on the N-atom. Furthermore, calculation of the spin distribution on the O- or N-protonated one electron reduced resorufins (Figure 6.4[b and c]) show a larger delocalization of the radical electron in the O-protonated form, suggesting it to be more stable than the N-protonated form. This is confirmed by calculation of the final heat of formation of the O- and N-protonated resorufin radicals respectively (Table 6.5), showing O-protonation to be 15.1 kJ/mol energetically favourable over N-protonation. Similar calculations for the one electron reduced 7-alkoxyresorufins provided the same results (data not shown), demonstrating the charge and spin distribution and thus the protonation to be independent of the ether side chain.

6.4.4 Redox cycling catalyzed by membrane incorporated NADPH-cytochrome reductase

In an additional experiment the redox cycling of resorufin by membrane incorporated reductase was studied. Upon membrane incorporation of the reductase by cholate dialysis, recovery of the cytochrome *c* reducing activity was over 95 %. Expressed per unit of cytochrome *c* reducing activity, membrane incorporation of the reductase showed no effect on ethoxyresorufin dependent redox cycling (Table 6.6). The resorufin dependent redox cycling, however, appeared to be significantly ($P<0.001$) higher when catalyzed by membrane incorporated NADPH-cytochrome reductase.

The explanation underlying the observed difference in resorufin redox cycling activity between solubilized and membrane incorporated NADPH-cytochrome reductase was investigated in a kinetic study. The data from this experiment are summarized in Table 6.7. The results clearly demonstrate that the higher resorufin redox cycling activity by membrane incorporated reconstituted reductase is caused by a significant

Table 6.6: Redox cycling of resorufin (50 μM final concentration) and ethoxyresorufin (4.5 μM final concentration) by solubilized and membrane incorporated NADPH cytochrome reductase.

substrate	reductase	redoxcycling (nmol NADPH oxidized. min^{-1}. unit $reductase^{-1}$)	% of solubilized activity
resorufin	solubilized	105 ± 1	100
	membrane incorporated	174 ± 4 *	168 ± 4 *
ethoxyresorufin	solubilized	183 ± 6	100
	membrane incorporated	188 ± 6	103 ± 7

Activity is expressed per unit of cytochrome c reducing activity of the two preparations. The values represent the mean ± SEM of five experiments.

* = significantly different from the solubilized reductase activity at P<0.001.

Table 6.7: Kinetic data from Lineweaver-Burk plots for the redox cycling of resorufin by solubilized and and membrane incorporated NADPH cytochrome reductase.

reconstituted system	apparent K_m (μM)	V_{max} (nmoles NADPH oxidized $.min^{-1}$ $.unit\ reductase^{-1}$)
solubilized reductase + PC di-12:0 vesicles	153 ± 13	512 ± 39
membrane incorporated reductase	107 ± 8*	571 ± 30

Redoxcycling was essentially measured as described in 'Materials and methods'. Redoxcycling by solubilized NADPH-cytochrome reductase was measured in the presence of PC di-12:0 vesicles. Lipid vesicles were added upto the reductase/phospholipid ratio used in the membrane incorporated system. The values represent the mean ± SEM of six experiments.

* = significantly different from the value for solubilized reductase at P<0.02

decrease in the apparent K_m for the resorufin ($P<0.02$), whereas the V_{max} is unaffected. With the assumption that only the protonated form of the resorufin contributes significantly to the resorufin redox cycling, it follows from the observed difference in apparent K_m that the concentration of the protonated resorufin in the membrane environment is 1.4 times its concentration in the bulk waterphase.

6.5 Discussion

The present study describes both experimental and theoretical data on the redox cycling of resorufins by NADPH-cytochrome reductase. Based on the outcomes of these studies a scheme for the redox cycling of resorufin at physiological pH can be presented (Figure 6.5) that differs from the one recently published in the literature by Dutton and Parkinson [4].

Redoxcycling of resorufin was shown to be pH dependent. Due to its pK_a determined to be 5.7, at physiological pH (pH 7.1-7.7) the compound is mainly (96-99%) present in its deprotonated O-anionic form. Based on further experimental data and the results from AM1 molecular orbital calculations presented in this study, it can be concluded that the protonated resorufin and not this O-anionic form is the one active in redox cycling. This conclusion emerges from the facts that i) redox cycling of resorufin increases with decreasing pH in spite of a lower activity of the NADPH-cytochrome reductase at these lower pH values, ii) the E_{LUMO} for the O-anionic form was positive and thus significantly higher than the negative E_{LUMO} for the protonated resorufin and iii) one electron reduction of the protonated form was calculated to be energetically favoured by 363.5 kJ/mol compared to one electron reduction of the resorufin O-anion.

In addition, AM1 molecular orbital calculations confirmed that protonation of the intramolecular semiquinoneimine moiety has to occur before a second one electron reduction is likely to take place. This is in agreement with an assumption made by Dutton and Parkinson [4]. Up to now, the literature data on the protonation of benzosemiquinoneimines show controversy as to whether the semiquinoneimines become protonated on their N- or O-atom [4,13,14]. Charge and spin distributions for N-acetyl-p-benzoquinoneimine were suggested, that located the charge and the unpaired electron on the O- and N-atom respectively,

suggesting protonation to take place at the O-atom [13]. On the other hand Fischer *et al.* [14] proposed the N-acetyl-p-benzosemiquinoneimine to become protonated at the N-atom. Dutton and Parkinson [4] proposed protonation of the intramolecular semiquinoneimine moiety of the resorufin radical anion to take place at the N-atom. In the present study however, the charge distributions, calculated for the one electron reduced resorufin, together with the spin distributions and the final heats of formation calculated for the O- or N-protonated form of the one electron reduced resorufin, indicate that the O-atom is the one most likely to become protonated.

The results of the present study demonstrate that calculation of the energy of the molecular orbital into which an electron will be placed upon redox cycling (E_{LUMO} for non-radicals), might be used as a tool to predict the capacity of a compound to take part in redox cycling reactions. However, affinity of the NADPH-cytochrome reductase for the compound, as well as the capacity of the one or two electron reduced xenobioticum to pass the electron(s) on to molecular oxygen, will also represent factors controlling redox cycling activities [13,15]. Obviously, possible correlations between calculated parameters (E_{LUMO} or ΔHF) and redox cycling of other xenobiotics than the resorufins, remains an interesting topic for future research.

It is also demonstrated in the present study that for resorufin at a given concentration the redox cycling by solubilized NADPH-cytochrome reductase was significantly lower than the activity by membrane incorporated NADPH-cytochrome reductase. The results of kinetic studies, using membrane incorporated reductase and solubilized reductase in the presence of PC di-12:0 vesicles, demonstrated this to be caused by a reduced apparent K_m for resorufin for the membrane incorporated enzyme. The V_{max} values for both systems appeared to be the same, but they were higher than for solubilized reductase without added phospholipid vesicles (Table 6.7). This higher V_{max} in the presence of phospholipids might be caused by an improved electron transfer from NADPH-cytochrome reductase to resorufin. For the cytochrome P450 enzyme system, which includes NADPH-cytochrome reductase, phospholipids have also been reported to affect electron transfer from NADPH-cytochrome reductase to cytochrome P450 [16-19].

The observed difference in apparent K_m for resorufin for membrane incorporated and solubilized reductase might originate from the fact that in the apolar membrane environment the amount of the protonated, redox cycling active form of resorufin is increased compared to its concentration in the bulk water phase. This could be the result of either partitioning of resorufin into the membrane or an effect of the membrane on the protonation equilibrium of resorufin in favour of the more apolar protonated form or a combination of both phenomena. The effect can not be ascribed to a change in proteinconformation of NADPH-cytochrome reductase upon membrane incorporation because the redox cycling of ethoxyresorufin was not affected by membrane incorporation. Furthermore, the fact that ethoxyresorufin redox cycling is not affected by membrane incorporation of the reductase also implies that the positive effect of membrane incorporation on the redox cycling of resorufin can not be solely ascribed to the partitioning of the substrate into the membrane, but must at least in part be caused by a shift in the protonation equilibrium of resorufin. In other literature reports it has often been assumed that membrane surroundings may influence substrate availabilities of apolar xenobiotics for biotransformation enzymes. Especially with respect to the cytochrome P450 enzyme system the membrane has often been regarded as the place where apolar substrates might accumulate [18,20-23]. Experimental evidence for this assumption was first provided in a study by Taniguchi and Pyerin [24]. They demonstrated that the apparent dissociation constant of benzphetamine for purified, solubilized cytochrome P450 was one order of magnitude larger than for purified, membrane incorporated cytochrome P450 and microsomal cytochrome P450. The results of the present study provide further evidence for the concept, mentioned above. In addition they demonstrate that membrane surroundings might even cause a shift in the protonation equilibrium of a xenobiotic compound, in favour of its most apolar form.

Such membrane induced shifts in protonation equilibria, as demonstarted for resorufin in the present study, might also influence the conversion of other xenobiotics by membrane bound biotransformation enzymes, such as cytochromes P450, UDP-glucuronyltransferases and microsomal glutathion S-transferase. Clearly this will especially be of importance when there is a difference in the rate of conversion of protonated and deprotonated forms of the xenobiotic.

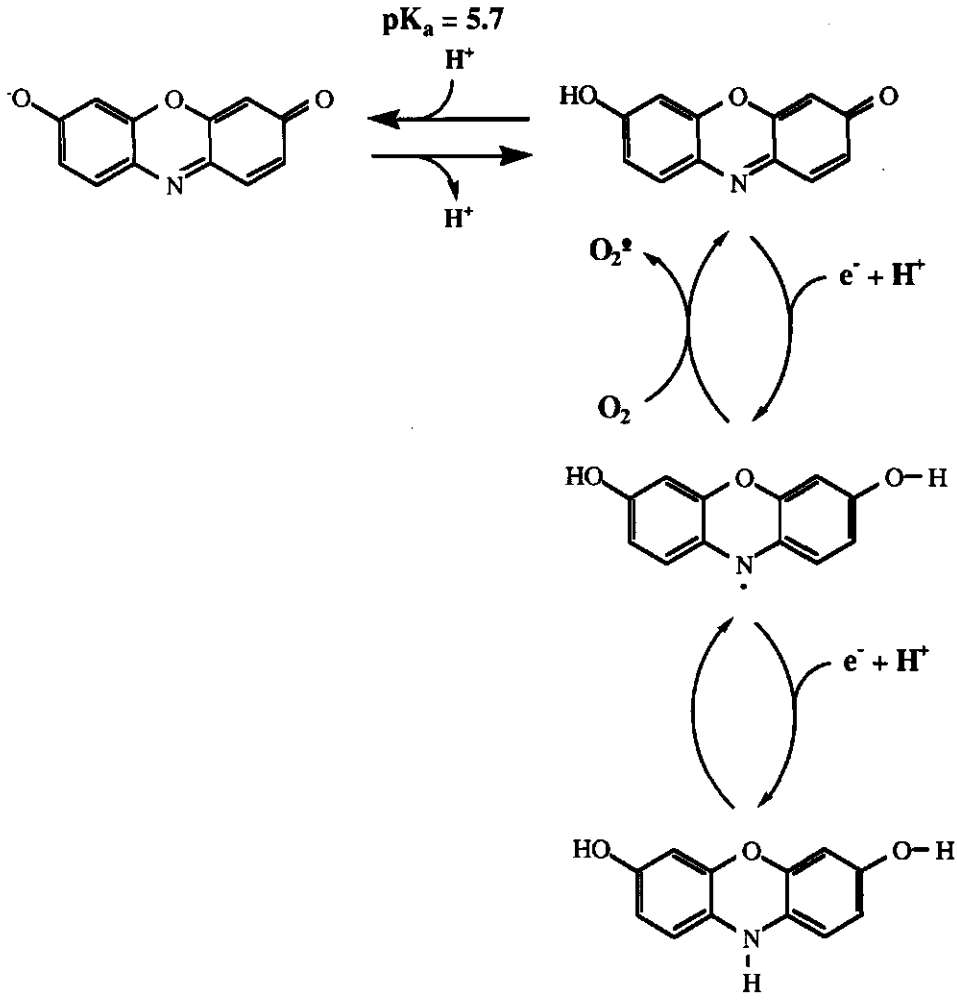


Figure 6.5: Reaction scheme for the NADPH-cytochrome reductase catalyzed redox cycling of resorufin based on the results of the present study.

The unpaired electron is situated on the atom with the highest spin density. The actual spin distribution is depicted in Figure 6.3.

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Chapter 7

NADPH-cytochrome reductase catalysed redox cycling of 1,4-benzoquinone; hampered at physiological conditions, initiated at increased pH values.

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7.1 Summary

In the present study the inability of 1,4-benzoquinone to support NADPH-cytochrome reductase catalyzed redox cycling was investigated. The results obtained demonstrate that NADPH-cytochrome reductase is able to initiate a rapid two electron reduction of 1,4-benzoquinone resulting in formation of the hydroquinone. The intermediate one electron reduced semiquinone form does not pass its electron on to molecular oxygen, i.e. giving rise to redox cycling, but is reduced by a second electron, either by NADPH-cytochrome reductase upon protonation of the semiquinone or through disproportionation, both giving rise to the two electron reduced hydroquinone. At pH values below the pK_a of the hydroquinone, the electrons of the hydroquinone are also not passed on to molecular oxygen due to efficient protonation. However, at pH values around or above the pK_a (9.85) of the two electron reduced hydroquinone form, significant redox cycling activity is observed in a 1,4-benzoquinone containing incubation. Further experiments demonstrate a similarity in both the concentration and pH dependence of 1,4-benzoquinone or 1,4-hydroquinone supported NADPH-cytochrome reductase catalyzed redox cycling. From these observations it is concluded that 1,4-benzoquinone is able to redox cycle from its deprotonated two electron reduced hydroquinone form, but only at relatively high pH values.

Together the data provide insight into why the NADPH-cytochrome reductase catalyzed redoxcycling of 1,4-benzoquinone is inhibited at physiological conditions, but initiated at increased pH values.

7.2 Introduction

The toxicity of quinones is generally ascribed to their capacity to bind covalently to cellular nucleophilic macromolecules, and/or to their capacity to support a process called redox cycling [1-3]. The redox cycling of quinones is initiated by their one electron reduction catalyzed by flavin containing enzymes like NADPH-cytochrome reductase (EC 1.6.2.4), NADH-cytochrome reductase (EC 1.6.2.2) or xanthine oxidase (EC 1.2.3.2) [4], resulting in formation of a reactive semiquinone radical. The semiquinone radical may pass its unpaired electron on to molecular oxygen, giving rise to the formation of reactive oxygen species and

regeneration of the quinone which can participate in a new redox cycle. In addition, upon protonation and a second single electron reduction of the semiquinone radical the two electron reduced deprotonated hydroquinone form arises. This form might also react with molecular oxygen passing on one or both of its electrons, resulting in formation of respectively superoxide anion radicals or hydrogen peroxide. The extent to which redox cycling may add to the toxicity of the benzoquinones varies with the substituent pattern of the quinone [3,5]. 1,4-Benzoquinone as well as mono-substituted 1,4-benzoquinone derivatives have been reported to redox cycle very poorly, or not at all [6]. The poor redox cycling of 1,4-benzoquinone has been ascribed to its very positive one-electron reduction potential in aqueous solutions, although the reason for poor redox cycling due to a relatively positive single electron reduction potential remained unclear [2,6,7]. Other investigators ascribed the lack of redox cycling ability of 1,4-benzoquinone to the slow reaction of its semiquinone radical with molecular oxygen ($k= 0.5\text{-}4.5 \times 10^4\ M^{-1}.s^{-1}$) [3,8,9,10].

Generally, factors not taken into consideration when studying the redox cycling capacities of the quinones in biological systems, are the protonation-deprotonation equilibria of their semiquinone and of their two electron reduced hydroquinone form, which are, however, well documented in the literature [11,12]. This, in spite of the fact that protonation can be expected to stabilize the negative charge resulting from the one or two electron reduction, thereby decreasing the possibility of a reaction of the one or two electron reduced form with molecular oxygen, i.e. redox cycling. That deprotonation is a requisite for electron transfer is a well established concept [13].

The objective of the present study was to investigate the lack of redox cycling of 1,4-benzoquinone and the importance of protonation equilibria of its one and two electron reduced forms for the inability of 1,4-benzoquinone to redox cycle in a NADPH-cytochrome reductase catalyzed reaction.

7.3 Materials and methods

7.3.1 Purification of NADPH cytochrome reductase.

NADPH-cytochrome reductase was purified from liver microsomes of female Wistar rats pretreated with phenobarbital (Brocacef b.v., Maarssen, The Netherlands) (0.1 % (w/v) in drinking water for 7 days), essentially as described by Yasukochi and Masters [14]. Emulgen 911 (Kao Corporation, Tokyo, Japan) was used instead of Renex 690, and all buffers contained 100 μM phenylmethanesulfonyl fluoride (Merck, Darmstadt, FRG) to inhibit protease activities. The preparation was made detergent-free as described before [15].

NADPH-cytochrome reductase activity was determined as described by Phillips and Langdon [16], using cytochrome *c* (Boehringer, Mannheim, FRG) as the final electron acceptor. One μmol cytochrome *c* reducing activity per minute was taken as the unit to quantify the NADPH-cytochrome reductase activity. Protein concentrations were measured using the method of Lowry *et al.* [17] using bovine serum albumin (Sigma, St Louis, MO, USA) as the standard. The final detergent-free preparation had a specific activity of at least 35 units/mg protein and was homogenous as judged by SDS-PAGE, carried out as described by Laemmli [18].

7.3.2 Redox cycling of benzoquinones.

1,4-Benzoquinone, 1,4-hydroquinone and tetramethyl-1,4-benzoquinone (duroquinone) were purchased from Aldrich (Steinheim, FRG). The redox cycling of benzoquinones was measured at room temperature in 0.1 M potassium phosphate buffer pH 7.5 (unless indicated otherwise) containing 0.15 mM NADPH (final concentration). The desired concentrations of quinones were added as 1% (v/v) of a 100 times concentrated, freshly prepared solution in dimethyl sulfoxide. Reactions were started by addition of 0.13 units of NADPH-cytochrome reductase. NADPH oxidation was detected by measuring the absorption at 340 nm. Activities were corrected for NADPH-cytochrome reductase-independent NADPH oxidation which was generally between 0 and 30 % of the reductase dependent activity.

Production of H_2O_2 was measured as described by Werringloer [19]. Oxygen consumption was measured using a Clark oxygen electrode at 25 °C.

7.3.3 Molecular orbital computer calculations.
Molecular orbital calculations were performed as described before [15].

7.3.4 Statistical analysis of data
The results represent the mean ± standard error of the mean.

7.4 Results

7.4.1 Redox cycling of the 1,4-benzoquinones.
The redox cycling of 1,4-benzoquinone and tetramethyl-1,4-benzoquinone (used as a well-known redox active control) was investigated as a function of the benzoquinone concentration.

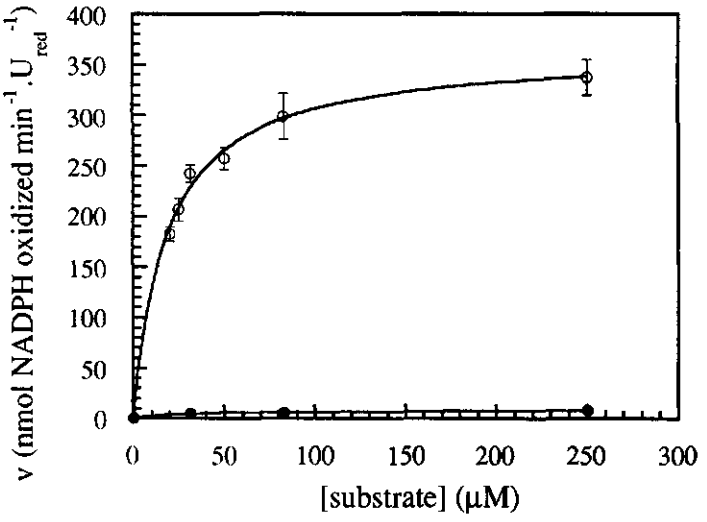

Figure 7.1: NADPH-cytochrome reductase catalyzed NADPH oxidation in the presence of tetramethyl-1,4-benzoquinone (o-o) or 1,4-benzoquinone (●-●) at pH 7.5 and increasing concentration of the benzoquinone.

NADPH-oxidation was measured as described in 'Materials and methods'. The results represent the mean ± standard error of the mean of three experiments.

From the results obtained (Figure 7.1) it follows that there is a significant difference in the extent to which the 1,4-benzoquinone and tetramethyl-1,4-benzoquinone redox cycle. Whereas redox cycling of 1,4-benzoquinone itself was hardly detectable at pH 7.5, substitution with four electron donating substituents (tetramethyl-1,4-benzoquinone)

resulted in increased rates of redox cycling. The apparent K_m and V_{max} for the NADPH-cytochrome reductase catalyzed redox cycling of tetramethyl-1,4-benzoquinone derived from the data presented in Figure 7.1 were respectively 19±1 μM and 367±22 nmol NADPH oxidized.min^{-1}.U_{red}^{-1}.

In the presence of superoxide dismutase (0.1 mg/ml) at physiological pH values redox cycling of 1,4-benzoquinone was still not observed, indicating that elimination of superoxide anions from the incubation medium does not result in a shift of the equilibrium semiquinone + oxygen ⇔ quinone + superoxide anion to the right, initiating possibilities for redox cycling.

7.4.2 Single electron reduction potentials of the 1,4-benzoquinones.

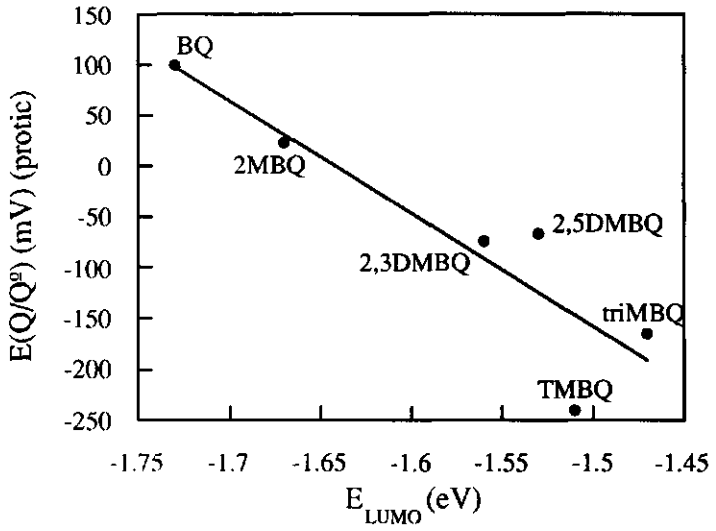

Figure 7.2: Relation between the single electron reduction potential (E(Q/Q•)) for a series of 1,4- benzoquinones in aqueous solution and their calculated E_{LUMO}. The correlation coefficient was 0.909.

The one electron reduction potentials in aqueous solution of the Q/Q• couples, indicated here as E(Q/Q•), were taken from Chambers [11]. The E_{LUMO} values were calculated as described in 'Materials and methods'. BQ = 1,4-benzoquinone; MBQ= methyl-1,4-benzoquinone; 2,3DMBQ = 2,3-dimethyl-1,4-benzoquinone; 2,5DMBQ = 2,5-dimethyl-1,4-benzoquinone; triMBQ = trimethyl-1,4-benzoquinone; TMBQ = tetramethyl-1,4-benzoquinone

Previous studies reported in the literature, suggest a relationship between electronic affinities, polarographic single electron reduction potentials and the energy (E) of the lowest unoccupied molecular orbital (LUMO) of a molecule [11,20,21,22]. The LUMO is the molecular orbital into which the electron will be positioned upon one electron reduction. To investigate whether the non redox cycling of 1,4-benzoquinone could be due to a deviating single electron reduction potential compared to the single electron reduction potential of tetramethyl-1,4-benzoquinone, the E_{LUMO} values of these and additional 1,4-benzoquinones were calculated and compared to data available in the literature [11,22] for their one electron reduction potentials. The results obtained (Figure 7.2) demonstrate that the calculated E_{LUMO} values correlate with the single electron reduction potential of the benzoquinones (correlation coefficient = 0.909).The data in Figure 7.2 also demonstrate that the one electron reduction potential of 1,4-benzoquinone is higher than that of methyl substituted benzoquinones, suggesting that the reduction of 1,4-benzoquinone (higher $E_{1/2}$, lower E_{LUMO}) is energetically more favourable than that of tetramethyl-1,4-benzoquinone. From this it is concluded that the absence of redox cycling of 1,4-benzoquinone is unlikely to be due to an inability to accept electrons.

7.4.3 NADPH-cytochrome reductase catalyzed reduction of 1,4-benzoquinone.

In Figure 7.3 data are presented that confirm that the poor redox cycling of 1,4-benzoquinone is not due to its inability to accept electrons. Immediately following the addition of NADPH-cytochrome reductase to a 1,4-benzoquinone containing incubation a rapid decrease in NADPH is observed, which levels off after a short period of time (Figure 7.3[a]). During this rapid decrease in NADPH no oxygen consumption is observed (data not shown). The amount of NADPH oxidized varies stoichiometrically with the amount of 1,4-benzoquinone added (Figure 7.3[b]). From this observation it follows that two electrons are consumed per molecule of 1,4-benzoquinone. This implies that the 1,4-benzoquinone can be one-electron reduced by NADPH-cytochrome reductase, but instead of passing its electron on to molecular oxygen, the semiquinone radical either disproportionates or becomes quickly reduced by a second electron, both processes resulting in formation of the two electron reduced hydroquinone.

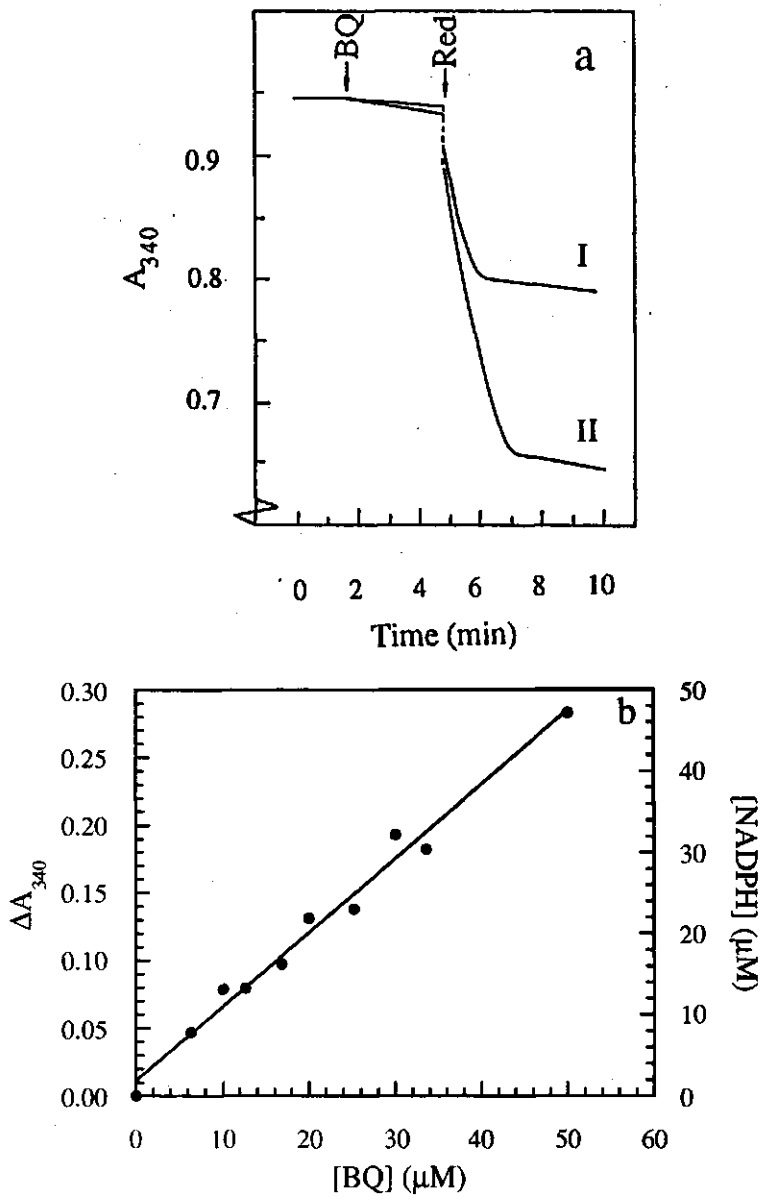

Figure 7.3: Time (a) and 1,4-benzoquinone concentration (b) dependent NADPH-cytochrome reductase catalyzed NADPH oxidation.

Incubation conditions were as described in 'Materials and methods'. NADPH oxidation was determined by monitoring the absorbance at 340 nm. At the arrows in Figure 3[a] respectively 1,4-benzoquinone (in two concentrations) and NADPH-cytochrome reductase were added to the incubation. In Figure 3[b] the relationship between the amount of 1,4-benzoquinone added and the amount of NADPH oxidized is depicted. The curve obtained fits the equation [NADPH] = 1.87 + 0.91.[HQ], correlation coefficient = 0.991.

7.4.4 Redox cycling of 1,4-benzoquinone at varying pH.

The lack of redox cycling of 1,4-benzoquinone might be ascribed to efficient protonation of either its one or its two electron reduced form. Thus, a shift in the pH of the incubation to a value around or above the pK_a of the one electron reduced semiquinone or the two electron reduced hydroquinone might result in increased possibilities for redox cycling of 1,4-benzoquinone. Therefore, the effect of varying pH of the incubation mixture on the NADPH-cytochrome reductase catalyzed redox cycling of 1,4-benzoquinone was investigated. Tetramethyl-1,4-benzoquinone was included in this experiment as a positive control, i.e. a compound capable of redox cycling at physiological pH values. From the data, presented in Figure 7.4, it follows that from pH 7.5 to 10.5 tetramethyl-1,4-benzoquinone demonstrates significant redox cycling. The activity observed parallels the activity of NADPH-cytochrome reductase, demonstrating reduced activity at higher pH values (Figure 7.4).

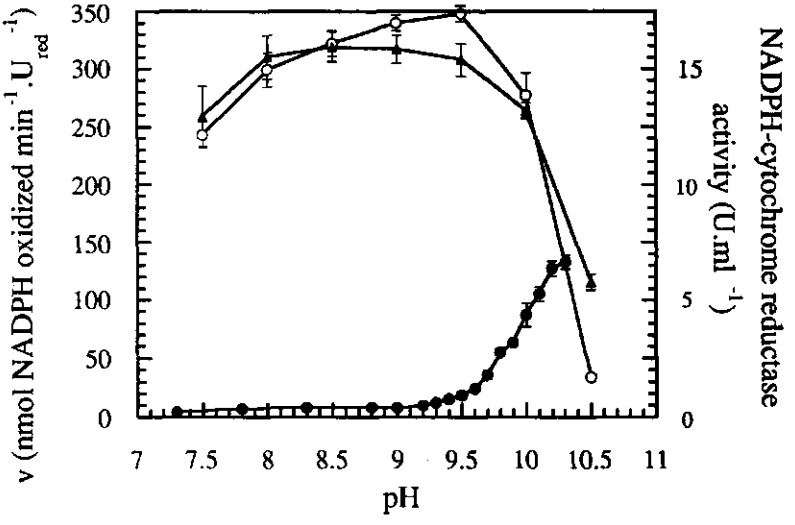

Figure 7.4: pH Dependence of the NADPH-cytochrome reductase catalyzed redox cycling of 1,4-benzoquinone (●-●) and tetramethyl-1,4-benzoquinone (o-o) and of the cytochrome c reducing activity of NADPH-cytochrome reductase (▲-▲).

Redox cycling activities in the presence of 1 μM 1,4-benzoquinone or 20 μM tetramethyl-1,4-benzoquinone and the cytochrome *c* reducing activity of NADPH-cytochrome reductase were determined as described in 'Materials and methods'. The results represent the mean ± standard error of the mean of two experiments.

For 1,4-benzoquinone a different pattern was observed. At pH values below 9.0 1,4-benzoquinone appeared unable to support redox cycling. However, an increase in the pH from 9.0 to 10.5 results in increased capacities for redox cycling in spite of the relative reduction in the activity of the NADPH-cytochrome reductase. Additional experiments demonstrated that the 1,4-benzoquinone supported NADPH oxidation was accompanied by stoichiometric oxygen consumption and H_2O_2 production (Table 7.1), indicating that the 1,4-benzoquinone supported NADPH oxidation at pH ≥ 9.0 indeed represents redox cycling.

Table 7.1: NADPH-cytochrome reductase catalyzed NADPH oxidation, H_2O_2 formation and O_2 consumption in the presence of 1,4-benzoquinone (BQ) or 1,4-hydroquinone (HQ) measured during the first two minutes of redox cycling at pH 10.0.

substrate	NADPH oxidation	H_2O_2 formation	O_2 consumption
	(nmol / 2 min.U_{red})	(nmol / 2 min.U_{red})	(nmol / 2 min.U_{red})
BQ (0.5 μM)	88 ± 3	64 ± 2	80 ± 1
HQ (0.5 μM)	74 ± 6	62 ± 4	84 ± 2

NADPH oxidation, H_2O_2 formation and O_2 consumption were measured as described in 'Materials and methods'. The results represent the mean ± standard error of the mean (n=3).

The data presented in Figure 7.4 also demonstrate that the redox cycling of 1,4-benzoquinone becomes significant around the pK_a value of its hydroquinone form reported to be 9.85 [22]. The pK_a of its semiquinone form has been reported to be 4.1 [22]. This result suggests that 1,4-benzoquinone redox cycles from its deprotonated hydroquinone form not from its semiquinone form. To investigate this possibility to a further extent additional experiments were performed using 1,4-hydroquinone as a substrate for NADPH-cytochrome reductase catalyzed redox cycling. The results from these experiments are presented in Figures 7.5[a] and 7.5[b]. Figure 7.5[a] demonstrates that the pH dependence of the 1,4-benzoquinone and 1,4-hydroquinone supported redox cycling activities are similar. The 1,4-hydroquinone supported redox cycling also becomes significant around the pK_a value of 1.4-hydroquinone, i.e. 9.85. Figure 7.5[b] demonstrates that, at pH 10.0, the NADPH-cytochrome reductase

catalyzed redox cycling of 1,4-benzoquinone and 1,4-hydroquinone also show a similar dependence on the substrate concentration.

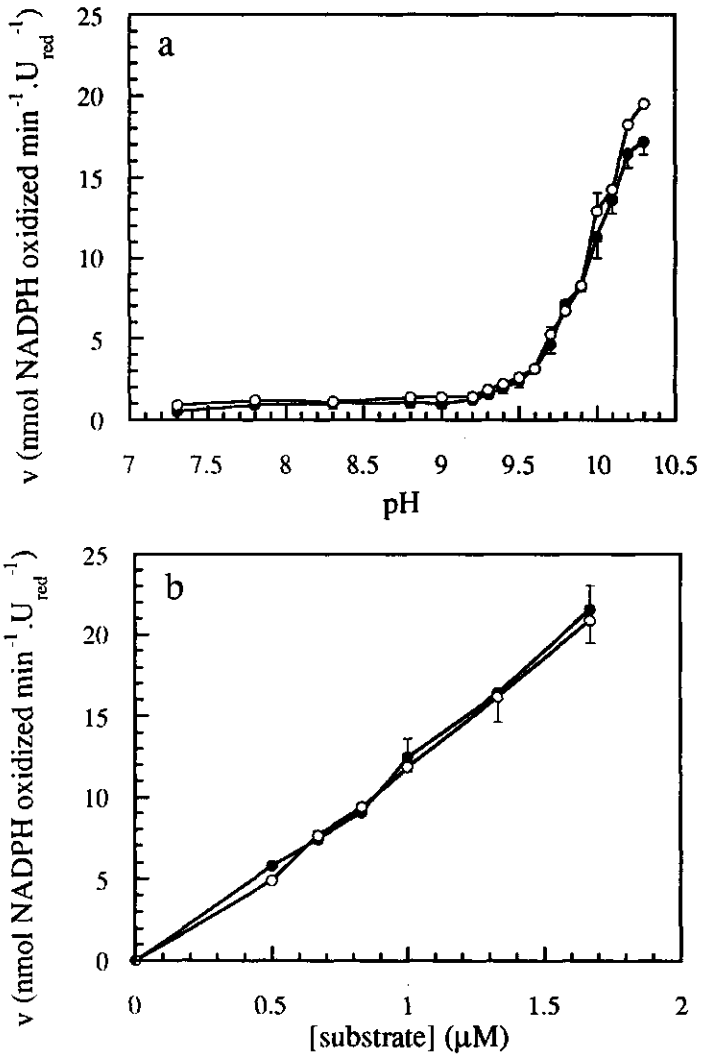

Figure 7.5: pH (a) and concentration (b) dependent NADPH-cytochrome reductase catalyzed redox cycling at pH 10.0 of 1,4-benzoquinone (●-●) and 1,4-hydroquinone (o-o).

Redox cycling was measured as described in 'Materials and methods'. The concentration of 1,4-hydroquinone and 1,4-benzoquinone used in Figure 7.5[a] was 1 μM. The results represent the mean ± standard error of the mean of two experiments.

Finally, the results presented in Figure 7.6 demonstrate that addition of the hydroquinone to an incubation without NADPH and without NADPH-cytochrome reductase (pH= 10.0), results in a stoichiometric consumption of oxygen. This implies an oxygen induced oxidation of the deprotonated hydroquinone.

Together the results support the conclusion that the redox cycling activites observed for 1,4-benzoquinone at pH values ≥ 9 can be ascribed to redox cycling of its deprotonated hydroquinone form.

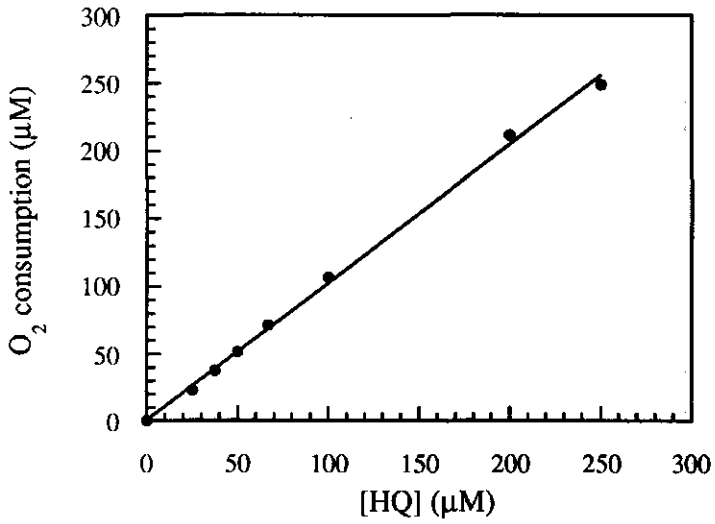

Figure 7.6: Oxygen consumption in an incubation without NADPH and without NADPH-cytochrome reductase upon addition of increasing concentration of 1,4-hydroquinone.

The curve obtained fits the equation O_2 consumption = 0.86 + 1.02.[HQ], correlation coefficient = 0.999. Oxygen consumption was measured as described in 'Materials and methods'. The results represent the mean ± standard error of the mean of two experiments.

7.5 Discussion

In the present study the inability of 1,4-benzoquinone to support NADPH-cytochrome reductase catalyzed redox cycling was investigated. Analysis of calculated molecular orbital parameters (E_{LUMO}) and the single electron reduction potentials of these and other quinone

derivatives, demonstrated that the non redox cycling of 1,4-benzoquinone can not be ascribed to a deviating single electron reduction potential of this molecule. Additonal experiments with 1,4-benzoquinone clearly demonstrated the two electron reduction of this compound by NADPH-cytochrome reductase resulting in stoichiometric NADPH oxidation and formation of the hydroquinone. This result is in accordance with results from an experiment described by Nishibayashi *et al.* [23] reporting stoichiometric NADPH oxidation upon addition of 1,4-benzoquinone to a microsomal incubation. The results of the present study demonstrate that this reduction is not accompanied by oxygen consumption or H_2O_2 formation but results in formation of the 1,4-hydroquinone. Based on these observations it must be concluded that the non redox cycling of 1,4-benzoquinone at pH 7.5 must be ascribed to the inability of both its one electron as well as its two electron reduced form to pass their electron(s) on to molecular oxygen. Taking into account the pK_a values reported for the semiquinone and hydroquinone form of 1,4-benzoquinone reported to be 4.1 and 9.85, respectively [22], it can be concluded that at pH 7.5 the semiquinone is present in its deprotonated form whereas the hydroquinone is present in its protonated form. Thus, the results obtained point at the inability of the deprotonated semiquinone and the protonated hydroquinone to pass electrons on to molecular oxygen. This conclusion is in line with the fact that the rate constant for the reaction of 1,4-semiquinone with molecular oxygen is at least four orders in magnitude lower than that of the tetramethyl substituted 1,4-semiquinone [9]. However, the rate constant for the protonation of the one electron reduced 1,4-benzoquinone is high compared to the rate constant for a reaction with molecular oxygen. Comparison of the rate constant for protonation of the semiquinone, reported to be 4×10^{10} $M^{-1}s^{-1}$ [24], to that reported for the reaction of the semiquinone with molecular oxygen ($0.5\text{-}4.5 \times 10^4$ $M^{-1}s^{-1}$) [8,10] shows that chances for protonation of the semiquinone radical are 10^6 times higher than chances for a reaction with molecular oxygen. When the protonated semiquinone is efficiently reduced by a second electron, i.e. removed from the incubation, this will shift the protonation equilibrium of the semiquinone in favor of the protonated form, even when the pH is above its pK_a. This explains why the semiquinone might become protonated and reduced by a second electron instead of passing on its electron to molecular oxygen, i.e. giving rise to redox cycling. Such a one electron reduction of the semiquinone to the

hydroquinone by NADPH-cytochrome reductase must be possible taking into account the one electron reduction potential of the semiquinone-hydroquinone couple of +459 mV [22] and the midpoint potentials of the four one electron steps in the NADPH-cytochrome reductase reported to be much lower, i.e. -110 mV, -270 mV, -290 mV and -365 mV [25]. In addition, disproportionation of the non-protonated semiquinone radical to result in formation of the quinone and the two electron reduced hydroquinone might provide an additional and/or alternative route for the NADPH-cytochrome reductase initiated formation of the two electron reduced hydroquinone.

Additional results of the present study demonstrate that with increasing pH 1,4-benzoquinone becomes capable of redox cycling. The actual pH at which this phenomenon occurs appears to be around the pK_a of the hydroquinone. From this result it is concluded that the redox cycling observed for 1,4-benzoquinone at increased pH values results from redox cycling of its deprotonated hydroquinone form, present at a significant concentration when the pH of the medium reaches the pK_a of the hydroquinone. This redox cycling implies electron transfer from the deprotonated hydroquinone to molecular oxygen, a reaction hampered at neutral pH by the fact that the one electron reduction potential for the semiquinone/hydroquinone couple might be too high, i.e. +459 mV [22], for an efficient one electron transfer of the hydroquinone to molecular oxygen. This because the reduction potential of the $O_2/O_2^{\bullet}$ couple has been reported to be between -155 and -330 mV [8,9,10,22]. However, the reduction potential of the semiquinone/hydroquinone couple will, in analogy to what is described for the two electron reduction potential of the quinone/hydroquinone couple, decline with increasing pH, facilitating the one electron transfer from the deprotonated hydroquinone to molecular oxygen. The fact that addition of superoxide dismutase did not inhibit the redox cycling of the (hydro)quinone at increased pH values (data not shown) excludes that the reaction proceeds by $O_2^{\bullet}$ as the oxidant. The stoichiometric oxygen consumption observed upon addition of the hydroquinone to an incubation without NADPH and without NADPH-cytochrome reductase further supports the O_2-catalyzed oxidation of the hydroquinone, as depicted in Figure 7.7.

In conclusion, the results of the present study demonstrate that the non redox cycling of 1,4-benzoquinone at pH 7.5 is mainly due to the low rate constant for the reaction of the 1,4-semiquinone with molecular oxygen

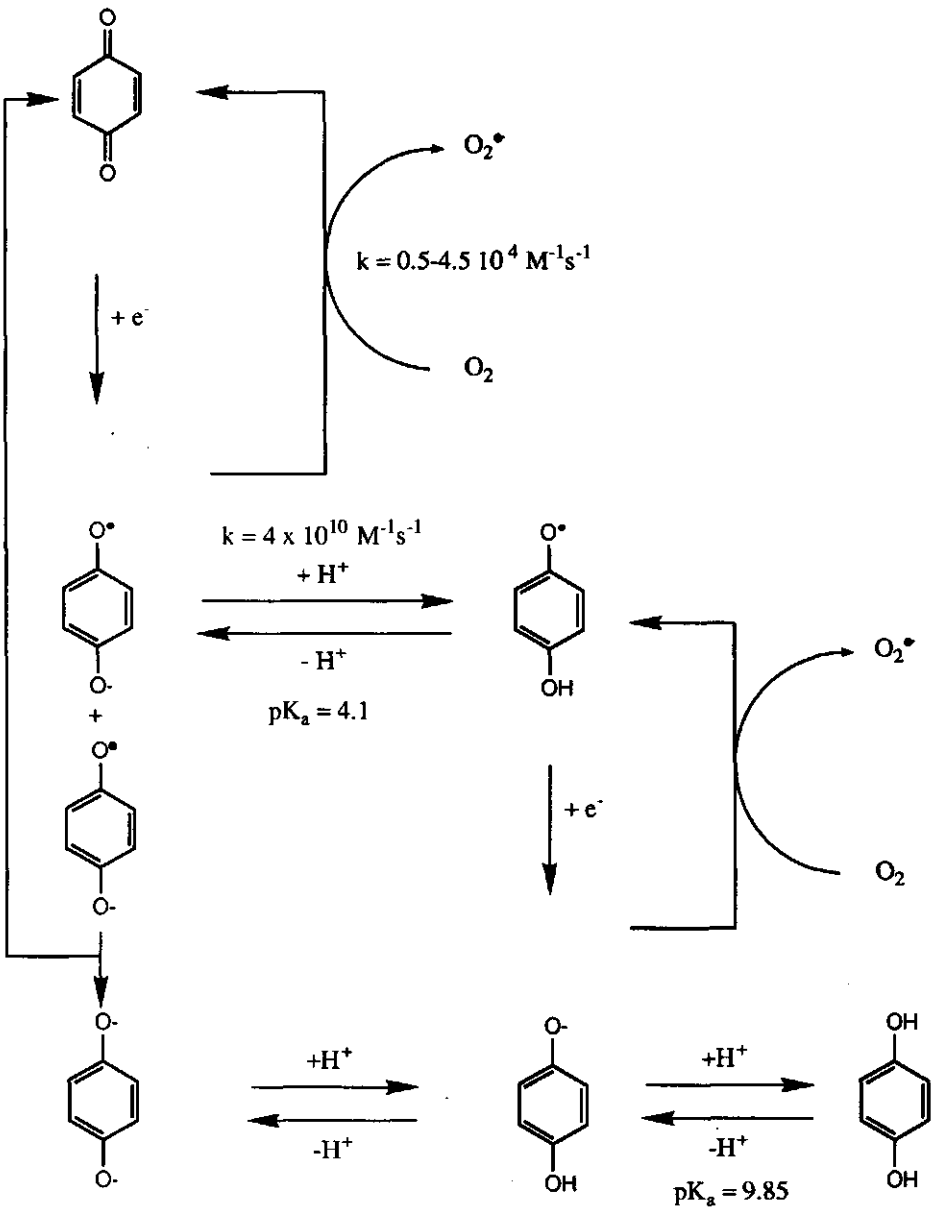

Figure 7.7: Schematic presentation of the pathway for redox cycling of 1,4-benzoquinone based on the results of the present study.

Kinetic data are derived from the literature [8,10,13]. For further explanation see text of the discussion section.

compared to the rapid protonation of the 1,4-semiquinone resulting in a form that can be efficiently reduced by a second electron and finally in the formation of the 1,4-hydroquinone. Due to its pK_a value of 9.85, at physiological pH the 1,4-hydroquinone becomes protonated which prevents redox cycling of this compound. Figure 7.7 schematically presents these characteristics of the 1,4-benzoquinone redox cycling. The results obtained support that under physiological conditions 1,4-benzoquinone-induced toxicity must result from its nucleophilic rather than its redox cycling capacities.

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Chapter 8

Summary and Conclusions

The cytochrome P450 enzyme system is involved in the metabolism and elimination of an almost unlimited number of endogenous and exogenous substrates. Biotransformation by cytochromes P450 plays a role in the conversion xenobiotics into more hydrophilic products. Generally, this process of biotransformation in which cytochrome P450 reactions take part, leads to elimination of the xenobiotic through urine and / or faeces although in some cases this process can also lead to the formation of more toxic metabolites. Because of its significant role in the conversion of numerous endogenous and exogenous compounds, a complete understanding of the cytochrome P450 enzyme system is of importance in toxicology, pharmacology, anesthesiology, pathology and other related biomedical fields.

Twenty-five years ago the phospholipids of the membrane of the endoplasmatic reticulum, to which the enzyme system is bound, appeared to play an important role in the *in vitro* cytochrome P450 enzyme system. Although the role of the membrane(phospholipids) in the cytochrome P450 system has been intensively studied since then, it has not resulted in a unanimous conclusion. The goal of this thesis was therefore, to gain further insight into the role of the membrane and membrane phospholipids in the cytochrome P450 system. Attention is thereby not only paid to the effects of the membrane and the phospholipids on cytochrome P450 enzymes but also to the effects on another important protein component of the enzyme system, namely NADPH-cytochrome reductase.

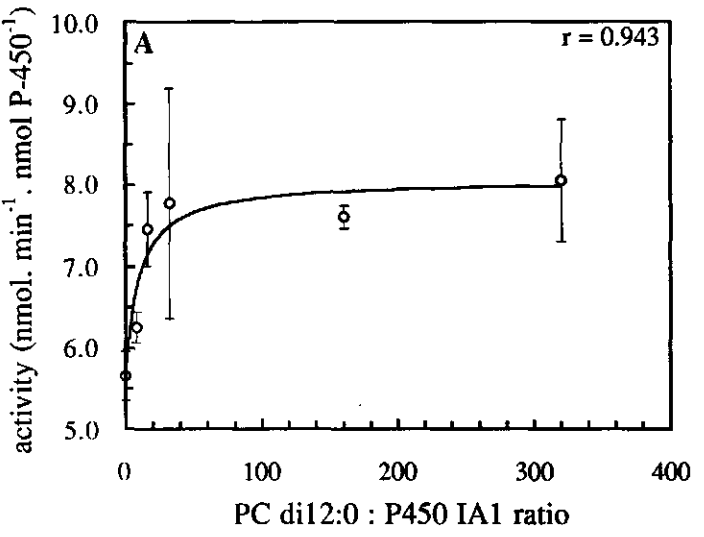
In the first two chapters the cytochrome P450 enzyme system and the membrane(phospholipids) of the endoplasmatic reticulum are described respectively. In **chapter 1A** the structural and catalytical properties of the cytochrome P450 enzyme system are briefly discussed. Special attention is paid to the occurence, multiplicity, induction, the structure of the individual protein components of the enzyme system and the catalytic cycle of cytochromes P450. The individual steps in this catalytic cycle are discussed in detail.

Chapter 1B deals with the structural aspects of the membrane phospholipids and the membrane of the endoplasmatic reticulum. In addition a brief description of the different types of reconstituted systems used in this thesis is given. Finally, the effects of phospholipids on the cytochrome P450 enzyme system, reported up to now in the literature,

and various current hypotheses for the stimulating effect of phospholipids are discussed briefly.

In **chapter 2** results are described that characterise the sensitivity of microsomal and isolated cytochrome P450 IA1 and IIB1 for an organic hydroperoxide; cumene hydroperoxide (CuOOH). These data provide information on the difference in the way of membrane incorporation of these two cytochrome P450 enzymes. Up to now, very little attention has been paid to possible differences in sensitivity of cytochromes P450 to conditions of oxidative stress. A difference in sensitivity for (hydro)peroxides between different forms of cytochrome P450, as demonstrated with CuOOH in the present study, can be of importance from a toxicological point of view. Especially in cases in which conversion by one cytochrome P450 enzyme results in detoxification of the substrate whereas another cytochrome P450 enzyme causes bioactivation of the substrate, a difference in sensitivity for conditions of oxidative stress can then result in a shift in the metabolite pattern.

Cytochrome P450 IIB1, embedded in the microsomal membrane is more sensitive towards CuOOH treatment than microsomal cytochrome P450 IA1. Purification of these enzymes and reconstitution in a system in which the proteins remain soluble results in a disappearance of the difference in CuOOH sensitivity between the two cytochrome P450 enzymes. Upon incorporation of cytochrome P450 IA1 and IIB1 into an artificial membrane, cytochrome P450 IIB1 again appears to be more sensitive towards CuOOH than cytochrome P450 IA1. Furthermore, the EC-50 values (effective cumene hydroperoxide concentration which causes 50% inhibition of cytochrome P450 dependent activities) in the microsomal and membrane incorporated reconstituted systems are comparable. Based on these results it is concluded that (1) the difference in sensitivity between cytochrome P450 IA1 and IIB1 towards treatment with CuOOH originates from a difference in the way these cytochrome P450 enzymes are incorporated into the membrane, that (2) the purification procedure does not affect the parameters determining the way of incorporation of the protein into the membrane and that (3) the way of membrane incorporation of cytochrome P450 enzymes in microsomal and reconstituted systems is comparable.



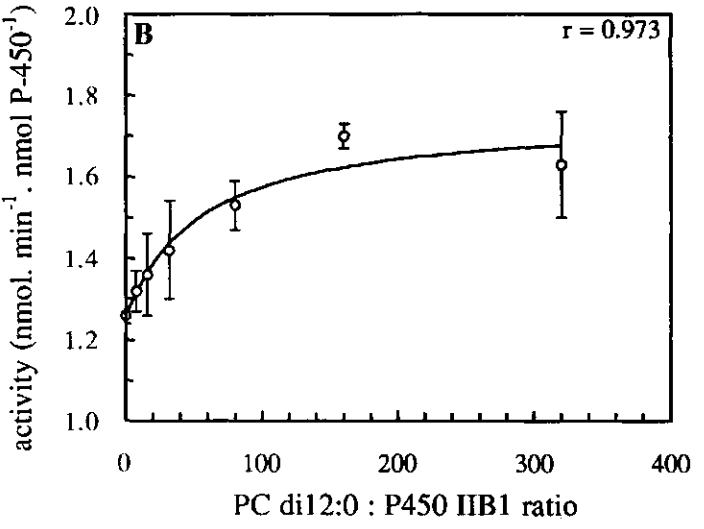


Figure 8.1: The effect of changes in the PC:P450 ratio on the O-dealkylation of ethoxycoumarin by cytochrome P450 IA1 and IIB1.

In **chapter 3** the effects of changes in the fatty acyl moiety of phosphatidylcholine (PC) from dilauroyl (di 12:0) to distearoyl (di18:0) on the kinetics of reconstituted cytochrome P450 IA1 and IIB1 were investigated. So far, studies on the effect of phospholipids on the kinetics of cytochrome P450 dependent reactions have focussed on one P450 enzyme or one phospholipid. Furthermore, mainly the effect on the activity at non-saturating substrate concentrations was investigated without paying much attention to the effect on the kinetic parameters K_m and V_{max}, of a cytochrome P450 catalysed reaction. An effect of phospholipids on for example the K_m could be of considerable importance especially because in living organism the substrate concentrations will generally be low. Furthermore, a different effect of phospholipids on the substrate's apparent K_m of different cytochrome P450 enzymes - converting the substrate to different metabolites - might not only affect substrate conversion rates but also the metabolite pattern.

The results presented in chapter 3 demonstrate that the V_{max} of the cytochrome P450 dependent O-dealkylation of alkoxyresorufins and ethoxycoumarin for both cytochrome P450 IA1 and IIB1 is two times higher in the PC di12:0 system compared to the PC di18:0 system. The effect of a change in the fatty acyl moieties on the K_m of the xenobiotic substrate however, appeared to be different for the two cytochrome P450 enzymes. For cytochrome P450 IA1 the K_m appeared to be lower in the PC di12:0 system compared to the PC di18:0 system whereas for cytochrome P450 IIB1 the K_m was lowest in the PC di18:0 system. Additional results demonstrated that the kinetic parameters were dependent on the PC : P450 ratio and that changing this ratio affected the kinetic parameters of cytochrome P450 IA1 and IIB1 in a different way.

The reason for the differential effect on the substrate apparent K_m was further investigated in a series of experiments in which the effect of PC di12:0 and PC di18:0 on individual steps of the catalytic cycle of cytochrome P450, like substrate binding, oxygen binding and rate of electron transfer, was studied. From these experiments it was concluded that the higher V_{max} in the PC di12:0 system, observed for both cytochrome P450 enzymes, was at least in part due to the higher affinity of cytochrome P450 for NADPH-cytochrome reductase in the PC di12:0 system. Furthermore, these experiments demonstrated that the different effect of a change in the fatty acyl moieties of PC on the K_m of cytochrome P450 IA1 and IIB1 did not result from a different effect on

substrate binding, oxygen binding and rate of electron transfer. This means that the differential effect on the K_m must result from an effect on one or more of the other steps in the catalytic cycle such as reductive oxygen splitting, substrate conversion and / or product release.

The results show that the effect of a change in the type of PC and / or the PC : P450 ratio on the kinetic parameters, K_m and V_{max}, is dependent on the cytochrome P450 enzyme used in the reconstitution. Furthermore, in contrast to what is generally assumed and based on results under non-saturating substrate conditions [1-4], the addition of PC appears to result for some cytochrome P450 enzymes in a decrease of the V_{max} in the reconstituted system. The results in chapter 3 also demonstrate that this is not reflected in lower but - in contrast - in higher conversion rates at non-saturating substrate concentrations (Figure 9.1), because the K_m is decreased simultaneously.

In **chapter 4** the existence of a preference - with respect to binding - of cytochrome P450 IIB1 for phospholipids with certain headgroups or fatty acyl moieties was investigated. The existence of "boundary" phospholipids (phospholipids which bind to cytochrome P450 with specificity and high affinity) for microsomal cytochrome P450 has been a topic for several studies. Nevertheless, little is known about this subject and unanimous conclusions have not been reached. It has been suggested that the composition of the membrane in the direct vicinity of cytochromes P450 is different from the rest of the membrane [5] and that specific interactions exist between cytochrome P450 and phosphatidylethanolamine (PE) [6] and phosphatidic acid (PA) [7].

The results in chapter 4 show that the apparent binding constant (K_d) of a cytochrome P-450 IIB1 - phospholipid complex is dependent on the degree of unsaturation of the phospholipid side chains; demonstrating a decrease in the K_d with increasing degree of unsaturation, but independent of the length of the acyl chains. In addition, the apparent K_d appeared to be dependent on the headgroup of the phospholipid molecule, showing a significantly higher K_d for PE di16:0 compared to PC di16:0, PS di16:0 and PI 16:0/18:1.

Translation of these results to the *in vivo* situation has to be done with caution because the results were obtained in a reconstituted system with isolated, solubilised cytochrome P450. In the membrane of the endoplasmatic reticulum other factors such as for example the presence

of other proteins can play an additional important role in the interaction of cytochrome P450 with phospholipids. Furthermore, the effect of the length and the degree of unsaturation of the fatty acyl chains on the K_d was determined for PC. It remains to be established whether for phospholipids with different headgroups similar influences of the length an degree of unsaturation of the acyl chains are observed. Investigations in this direction are however, seriously hampered by the fact that series of pure molecular species of PE, PS and PI are not commercially available.

In **chapter 5** the existence of specific phospholipid : protein interactions for NADPH-cytochrome reductase was investigated. Compared to cytochrome P450 very little attention has been paid to possible interactions between phospholipids and NADPH-cytochrome reductase and possible consequences of such interactions for the cytochrome P450 system. NADPH-cytochrome reductase is a very important component of the cytochrome P450 enzyme system and the stimulating effect of phospholipids on the rate of cytochrome P450 dependent reactions may in part originate from an effect on NADPH-cytochrome reductase resulting in a more efficient electron transfer to the cytochromes P450.

Based on the results from ^{31}P-NMR experiments and chemical analysis, it was concluded that NADPH-cytochrome reductase exhibits a preference for the negatively charged phospholipids phosphatidylserine (PS) and phosphatidylinositol (PI). In addition, experiments investigating the possible consequences of a special interaction of NADPH-cytochrome reductase with PS and PI demonstrated that (1) PS and PI had a significantly different effect on the DPH-PC dependent quenching of tryptophan fluorescence of NADPH-cytochrome redcutase compared to PE and PC and that (2) the V_{max} of cytochrome P450 IIB1 dependent O-dealkylation of pentoxyresorufin in the presence of 1:1 mixtures of PS:PC and PI:PC were respectively higher and lower compared to the V_{max} in the presence of a 1:1 mixture of PE:PC or PC alone. These phenomena might best be explained by a PS and PI induced specific change in the conformation of NADPH-cytochrome reductase. Regarding the fact that the specific interaction in both cases involves a negatively charged phospholipid suggest a possible role of the phospholipid charge. However, the fact that the effects of PS and PI on the V_{max} of the cytochrome P450 catalysed reaction are different demonstrates that phospholipid charge cannot be the only factor.

In **chapter 6** the redox cycling of 7-alkoxyresorufins and the product of their metabolism by cytochrome P450, resorufin, by NADPH-cytochrome reductase is investigated. Redox cycling is a process in which a substrate is 1-electron reduced, in this case by NADPH-cytochrome reductase. The electron is transfered to molecular oxygen and the substrate is returned to its initial state and can enter a new cycle. During this process reactive oxygen species are formed which can initiate lipidperoxidation and / or inactivate cytochrome P450. Especially in systems in which the NADPH-cytochrome reductase concentration is relatively high this process is a disturbing side-reaction, because it uses up reduction equivalents resulting only in the formation of reactive oxygen species which may cause protein inactivation and lipidperoxidation. In the reconstituted systems used in this thesis redox cycling can play an important role because the NADPH-cytochrome reductase : cytochrome P450 ratio is 15 to 30 times higher than in the *in vivo* situation. Furthermore, cytochrome P450 has been demonstrated to be very sensitive to lipidhydroperoxides - formed during lipidperoxidation - and reactive oxygen species.

The results of the present chapter demonstrate that at physiological pH alkoxyresorufins are much better substrates for redox cycling than resorufin. The inability of resorufin to stimulate redox cycling originates from the fact that at physiological pH resorufin exists mainly in its deprotonated form and this form is a much worse substrate for redox cycling than its protonated form. AM1 molecular orbital computer calculations demonstrated that the energy (E) of the lowest unoccupied molecular orbital (LUMO), i.e. the orbital into which the electron will be placed during redox cycling, of the deprotonated form is higher compared to the E_{LUMO} of the protonated form. Furhermore, one-electron reduction of the protonated form appeared to be energetically favorable by 363.5 kJ/mol over one-electron reduction of the deprotonated form. In addition, the computer calculations demonstrated that the one electron reduced resorufin is most likely to become protonated at the O-atom of the intramolecular semiquinone imine moiety before reduction by a second electron. Finally, it was demonstrated that incorporation of NADPH-cytochrome reductase into an artificial membrane results in an increased redox cycling activity of resorufin compared to solubilized NADPH-cytochrome reductase. This was explained by an increase in the protonated form in the membrane either by (1) favored partitioning of the

protonated form into the membrane or by (2) an effect of the membrane on the protonation equilibrium of resorufin in favor of the protonated form. This result points at the role of the membrane in concentrating apolar substrates of the cytochroom P450 : NADPH-cytochrome reductase system [8-10].

In **chapter 7** the use of AM1 MO calculations in predicting the ability of compounds to stimulate redox cycling, as demonstrated in chapter 6, was further investigated. Therefore, in addition to resorufins, the redox cycling ability of 1,4-benzoquinones was investigated. Quinones are toxic compounds that are often used in chemistry. They are also used in pharmacology, for example as anticancer drugs because of their toxic character. The mechanism through which quinones exert their toxic effects is believed to involve the covalent binding of quinones to cellular nucleophilic macromolecules and /or the quinone catalysed process of redox cycling. 1,4-Benzoquinone has been demonstrated to redox cycle very poorly. In the literature, the poor redox cycling of 1,4-benzoquinone has been ascribed to a very high 1-electron reduction potential. The results from chapter 7 demonstrate however, that, at physiological pH, 1,4-benzoquinone is quickly 2-electron reduced by NADPH-cytochrome reductase to form 1,4-hydroquinone. Instead of transfering its electron on to molecular oxygen, the 1-electron reduced semiquinone is protonated and subsequently reduced by a second electron. However, at pH>9 the 1,4-benzoquinone appears to be capable of stimulating redox cycling. Furthermore, the pH- and concentration-dependencies of redox cycling in a system with NADPH-cytochrome reductase between 1,4-benzoquinone and 1,4-hydroquinone are demonstrated to be similar. Based on these observations it was concluded that 1,4-benzoquinone is capable of redox cycling from its deprotonated, 2-electron reduced form at relatively high pH levels which ensures an adequate concentration of the deprotonated form. The results from chapters 6 and 7 demonstrate the importance of the protonation / deprotonation equilibrium of the 1- and 2-electron reduced forms in the redox cycling process.

In the paragraphs above the results of the study on the role of phospholipids in the cytochrome P450 : NADPH-cytochrome reductase system as it was executed for this PhD thesis are presented. Ever since 1968 [11] the role of phospholipids in this system has been a topic for

numerous studies in which many questions concerning this role have been answered but many new questions have also been raised. Unfortunately, this thesis does not provide the answers to all these new questions because the cytochrome P450 system is too complex and the number of different phospholipids and the effects they induce is too large. Answering these questions will require many, many years of additional research. The goal of this thesis was to investigate certain aspects of phospholipids and the cytochrome P450 enzyme system to which up to now little attention has been paid in order to gain further insight into the role of phospholipids in the cytochrome P450 enzyme system.

Altogether, the results of the experiments in this thesis present some new insightsin the role of phospholipids on the cytochrome P450 enzyme system. Furthermore, additional evidence for already existing hypotheses of the stimulating effect of phospholipids are also presented. The conclusions of the present thesis can be summarized as follows.

(1) Cytochromes P450 can differ in the way they are incorporated in the membrane which might result in differences in their sensitivity towards cumene hydroperoxide.

(2) The way of incorporation of cytochrome P450 enzymes in microsomal and reconstituted systems is comparable.

(3) Phospholipids influence the kinetic parameters, K_m and V_{max}, of cytochrome P450 catalysed reactions in reconstituted systems.

(4) The result of the effect phospholipids on the kinetic parameters of cytochrome P450 dependent reactions is not the same for all P450 enzymes.

(5) The affinity of cytochrome P450 for NADPH-cytochrome reductase in a reconstituted system is dependent on the fatty acyl moiety of the phospholipid added to the system.

(6) Phospholipids can decrease the apparent K_d of cytochromes P450 for their xenobiotic substrates.

(7) The apparent K_d of cytochromes P450 for phospholipids is dependent on the headgroup of the phospholipid and the degree of unsaturation of the fatty acyl chains but independent of the length of the acyl chains of the PC molecule.

(8) There is a specific interaction between NADPH-cytochrome reductase and the negatively charged phospholipids PS and PI.

(9) The membrane functions as a place where apolar substrates can accumulate thereby decreasing the apparent K_m.

(10) The membrane causes a shift in the overall protonation equilibrium of the substrate towards the protonated form.

In addition to these conclusions a number of other interesting phenomena have been observed that have no bearing on the role of phopsholipids in the cytochrome P450 enzyme system but are also worth mentioning again.

(1) The parameters determining the way of incorporation of cytochromes P450 in the membrane are not affected by the isolation procedure.

(2) AM1 molecular orbital calculations is a useful additional tool in investigating the redox cycling capacity of chemicals.

(3) The protonationequilibria of 1- and 2-electron reduced compounds play an important role in their redox cycling ability.

Finally, regarding the results of the experiments presented in this thesis one final conclusion must be added. The effect of phospholipids on the cytochrome P450 enzyme system is dependent on many factors such as the cytochrome P450 form, the fatty acyl moiety and headgroup of the phospholipid, the P450 : reductase ratio and the phospholipid : P450 ratio. Therefore, for a complete understanding of the mechanism(s) of action of phospholipids in the cytochrome P450 enzyme system, a detailed investigation of the effects of all phospholipids (and mixtures of phospholipids) on all P-450 forms at several P-450 : reductase and phospholipid : P-450 ratio's is necessary. This requires a vast amount of work although some of this work has already been done in these last twenty-five years. Comparison of these results is , however, difficult because of the different conditions used in these studies. It is therefore, advisable to come, in analogy to the nomenclature of cytochrome P-450, to standardized conditions for research in order for the results of different laboratories to be compared.

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Chapter 9

Samenvatting en conclusies

Het cytochroom P450 enzymsysteem is van belang voor de omzetting en uitscheiding van een bijna oneindige hoeveelheid xenobiotica en endogene substraten. De omzetting door het cytochroom P450 enzym systeem speelt een rol bij de conversie van xenobiotica in produkten die goed water oplosbaar zijn en het lichaam via urine en / of faeces kunnen verlaten. In veel gevallen leidt het proces van biotransformatie waar cytochroom P450 reacties deel van zijn tot eliminatie van het xenobioticum, maar in sommige gevallen kan dit proces ook leiden tot de vorming van een meer toxisch metaboliet. Door zijn belangrijke rol bij de omzetting van allerlei lichaamsvreemde en lichaamseigen stoffen is een volledig begrip van het cytochroom P450 enzym systeem van belang in de toxicologie, farmacologie, anesthesiologie, pathologie en andere gerelateerde biomedische gebieden.

Vijfentwintig jaar geleden bleek uit *in vitro* onderzoek dat de fosfolipiden van het membraan van het endoplasmatisch reticulum, waaraan het cytochroom P450 enzymsysteem gebonden is, een belangrijke rol heeft bij het functioneren van dit enzymsysteem. Sindsdien is deze rol uitvoerig onderzocht maar al dit werk heeft nog niet tot een eensluidende conclusie geleid omtrent de rol van membraanfosfolipiden in het cytochroom P450 systeem. Het doel van het in dit proefschrift beschreven onderzoek is het verkrijgen van meer inzicht in de rol van het membraan en membraanfosfolipiden bij het functioneren van het cytochroom P450 enzymsysteem. Hierbij wordt niet alleen aandacht besteed aan de effecten van het membraan en het fosfolipide op cytochroom P450 enzymen maar ook aan de effecten op een andere belangrijke eiwit component van het enzymsysteem, nl. het NADPH-cytochroom reductase.

In het eerste hoofdstuk wordt respectievelijk het cytochroom P450 enzymsysteem en het membraan(fosfolipide) van het endoplasmatisch reticulum nader beschreven. In **hoofdstuk 1A** worden in het kort de structurele en katalytische eigenschappen van het cytochroom P450 enzym systeem behandeld. Daarbij wordt aandacht besteed aan het voorkomen, de multipliciteit, inductie, de structuur van de verschillende eiwitcomponenten van het enzymsysteem en de katalytische cyclus van het cytochroom P450. De afzonderlijke stappen in deze katalytische cyclus worden nader beschreven.

Hoofdstuk 1B beschrijft de structurele aspecten van membraan fosfolipiden en het membraan van het endoplasmatisch reticulum en geeft daarnaast een beknopte beschrijving van de verschillende soorten gereconstitueerde systemen die in dit proefschrift gebruikt zijn. Tot slot worden in het kort de huidige inzichten omtrent de rol van fosfolipiden in het cytochroom P450 systeem beschreven en de momenteel geponeerde hypothesen voor het stimulerend effect van fosfolipiden op het enzymsysteem.

Hoofdstuk 2 levert via de resultaten omtrent de gevoeligheid van microsomaal en gezuiverd cytochroom P450 IA1 en IIB1 voor een organisch hydroperoxide; cumene hydroperoxide (CuOOH), informatie over de (on)gelijkheid van de wijze van membraanincorporatie van deze twee cytochroom P450 enzymen. Tot op heden, is er weinig aandacht besteed aan mogelijke verschillen in gevoeligheid tussen verschillende cytochroom P450 enzymen voor condities van oxidatieve stress. Vanuit een toxicologisch oogpunt kan een verschil in gevoeligheid voor (hydro)peroxides tussen verschillende cytochroom P450 enzymen, zoals gedemonstreerd voor CuOOH in deze studie, van belang zijn. Met name in het geval waarin een cytochroom P450 enzym zorgt voor detoxificering terwijl een ander cytochroom P450 enzym zorgt voor bioactivering, kan een verschil in gevoeligheid voor condities van oxidatieve stress mogelijk resulteren in een verschuiving in het metabolietpatroon en de toxicologische consequenties van de biotransformatie.

De resultaten van de experimenten beschreven in hoofdstuk 2 laten zien dat microsomaal cytochroom P450 IIB1 gevoeliger is voor behandeling met CuOOH dan microsomaal cytochroom P450 IA1. Wanneer deze enzymen gezuiverd worden en gereconstitueerd in een systeem waarin de eiwitten zich vrij in een waterige oplossing bevinden dan verdwijnt echter het verschil in CuOOH gevoeligheid tussen de twee cytochroom P450 enzymen. Incorporatie van cytochroom P450 IA1 en IIB1 in een kunstmatige membraan heeft tot gevolg dat cytochroom P450 IIB1 wederom gevoeliger wordt voor CuOOH dan cytochroom P450 IA1. Het blijkt dat de EC-50 waarden (de CuOOH concentratie waarbij de cytochroom P450 afhankelijke activiteit nog maar 50% is van de oorspronkelijke activiteit) in het microsomale en het gereconstitueerde membraan gebonden systeem vergelijkbaar zijn. Op basis van deze resultaten werd geconcludeerd dat (1) het verschil in gevoeligheid tussen

cytochroom P450 IA1 en IIB1 voor behandeling met CuOOH wordt veroorzaakt door een verschil in de wijzen van inbouw van de twee cytochroom P450 enzymen in de membraan, dat (2) de zuiveringsprocedure de parameters die de inbouw van het eiwit in de membraan bepalen niet beïnvloedt en dat (3) de manier van inbouw van cytochroom P450 enzymen in het microsomale en het gereconstitueerde vergelijkbaar is.

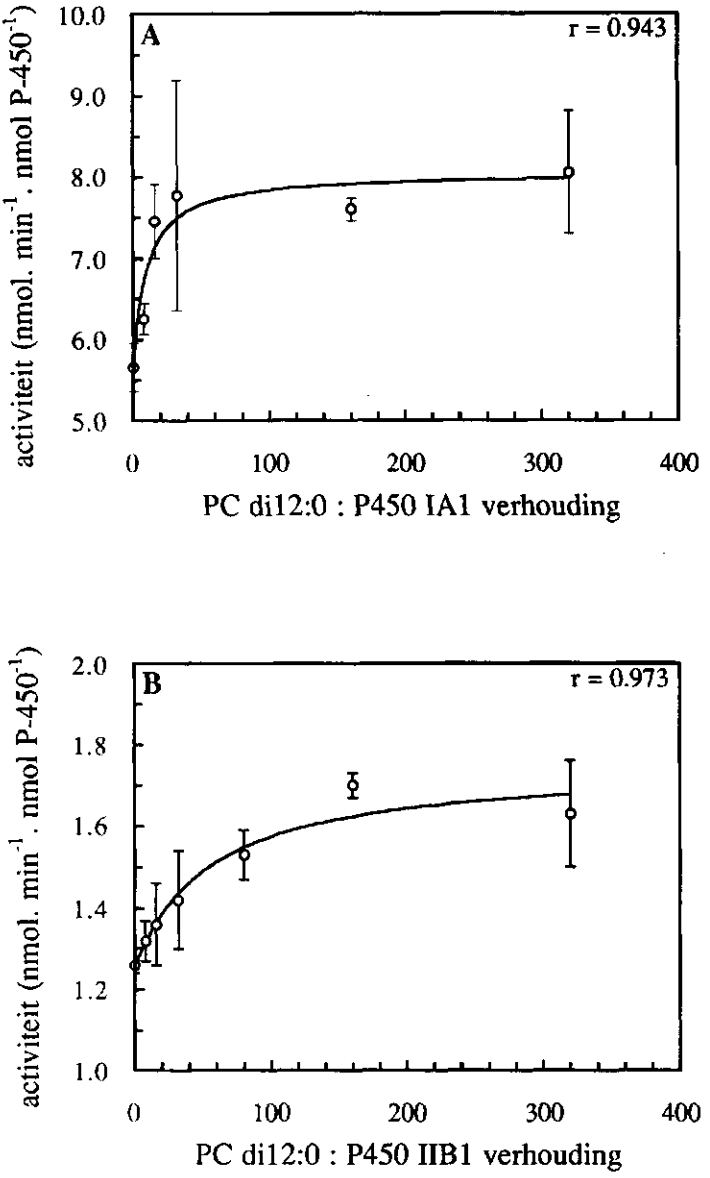
In **hoofdstuk 3** wordt gekeken naar het effect van een verandering in de vetzuurstaartsamenstelling van fosfatidylcholine (PC) van dilauroyl (di12:0) naar distearoyl (di18:0) op het kinetisch gedrag van cytochroom P450 IA1 en IIB1. Tot dusver zijn studies naar het effect van fosfolipiden op de kinetiek van cytochroom P450 afhankelijke reacties vooral gericht geweest op één P450 enzym of maar één of enkele fosoflipide. Met name is er gekeken naar de activiteit (onder niet-verzadigde substraatconcentraties) en in slechts weinig gevallen naar het effect op de kinetische parameters K_m en V_{max}. Een effect van de fosfolipiden op bijvoorbeeld de K_m van cytochroom P450 voor substraat kan echter van belang zijn met name omdat in het lichaam de substraatconcentraties over het algemeen laag zijn. Bovendien zou een verschillend effect van fosfolipiden op de K_m van verschillende cytochroom P450 enzymen - die een substraat omzetten tot meerdere produkten - niet alleen kunnen leiden tot een effect op de omzettingssnelheid maar ook op het metabolietpatroon.

In dit hoofdstuk wordt beschreven dat de V_{max} van cytochroom P450 afhankelijke O-dealkylering van alkoxyresorufines en ethoxycoumarine voor beide cytochroom P450 enzymen twee maal zo hoog is in het PC di12:0 systeem vergeleken met het PC di18:0 systeem. Het effect van een verandering in de vetzuurstaarten op de substraat afhankelijke K_m bleek echter verschillend te zijn voor de twee cytochroom P450 enzymen. Voor cytochroom P450 IA1 bleek de K_m in het PC di12:0 systeem lager te zijn dan in het PC di18:0 systeem terwijl voor cytochroom P450 IIB1 de K_m in het PC di12:0 systeem hoger is dan in het PC di18:0 systeem. Additionele resultaten toonden aan dat de kinetische parameters afhankelijk waren van de PC : P450 verhouding en dat veranderingen in deze verhouding de kinetische parameters van P450 IA1 en IIB1 verschillend beïnvloeden.

De reden voor dit verschillende effect op de substraat afhankelijke K_m werd onderzocht in een serie experimenten waarin de invloed van PC di12:0 en PC di18:0 op verschillende stappen in de katalytische cyclus van cytochroom P450 zoals substraatbinding, zuurstofbinding en de snelheid van elektron overdracht werd bestudeerd. Uit deze experimenten bleek dat de voor beide cytochroom P450 enzymen waargenomen hogere V_{max} in het PC di12:0 systeem vergeleken met het PC di18:0 systeem ten minste ten dele veroorzaakt wordt door een hogere affiniteit van cytochroom P450 voor NADPH-cytochroom reductase in het PC di12:0 systeem. Deze experimenten lieten echter ook zien dat het verschillende effect van PC di12:0 en PC di18:0 op de K_m van cytochroom P450 IA1 en IIB1 voor ethoxycoumarine niet bepaald wordt door een verschillend effect op de substraatbinding, zuurstofbinding en de snelheid van elektron overdracht. Dit houdt in dat het verschillend effect van de fosfolipiden op de K_m een gevolg is van een effect op één of meerdere van de andere stappen in de katalytische cyclus zoals zuurstofsplitsing, substraatomzetting en / of produktafsplitsing.

De resultaten laten zien dat het effect van een verandering in het type fosfatidylcholine en / of in de PC : P450 verhouding op de kinetische parameters, K_m en V_{max}, afhankelijk is van het cytochroom P450 enzym gebruikt bij de reconstitutie. Bovendien blijkt de toevoeging van fosfatidylcholine voor sommige cytochroom P450 enzymen te resulteren in een verlaging van de V_{max} in een gereconstitueerd systeem, dit in tegenstelling tot wat over het algemeen wordt aangenomen op basis van resultaten onder omstandigheden van niet-substraat-verzadiging [1-4]. Dit resulteert echter niet in een verlaging van de omzettingssnelheid onder niet-verzadigde substraat concentraties maar in een verhoging (figuur 1) omdat tegelijkertijd ook de K_m wordt verlaagd.

In **hoofdstuk 4** is onderzocht of er een voorkeur bestaat - wat betreft de binding - van cytochroom P450 IIB1 voor fosfolipiden met een bepaalde kopgroep of vetzuursamenstelling. Het bestaan van zogenaamde "boundary" fosfolipiden (=fosfolipiden die specifiek en met hoge affiniteit binden aan cytochroom P450) bij microsomaal cytochroom P450, is onderwerp geweest van verschillende studies.



Figuur 9.1: Het effect van veranderingen in de PC:P450 verhouding op de O-dealkylering van ethoxycoumarine door cytochroom P450 IA1 en IIB1.

Desondanks is er nog maar weinig bekend en men is nog niet tot een eensluidende conclusie gekomen. Er is geopperd dat de samenstelling van de membraan in de direkte omgeving van cytochroom P450 anders is dan de samenstelling van de rest van de membraan [5] en dat er specifieke interacties bestaan tussen cytochroom P450 en phosphatidyl-ethanolamine (PE) [6] en phosphatidezuur (PA) [7].

De resultaten in hoofdstuk 4 laten zien dat de bindingsconstante (K_d) van cytochroom P450 IIB1 voor fosfolipiden afhankelijk is van de graad van onverzadigdheid van de vetzuurstaarten; er werd een tendens waargenomen van een afnemende K_d met toenemende graad van onverzadigdheid. Ook bleek de bindingsconstante afhankelijk te zijn van de kopgroep van de fosfolipiden met een significant hogere K_d voor PE di16:0 in vergelijking tot PC di16:0, PS di16:0 en PI16:0/18:1. De bindingsconstante bleek onafhankelijk van de lengte van de acylketens in PC.

Vertaling van deze resultaten naar de *in vivo* situatie is uiterst riskant omdat er bij deze experimenten gebruik gemaakt is van een *in vitro* model systeem. In het membraan van het endoplasmatisch reticulum kunnen andere factoren zoals bijvoorbeeld de aanwezigheid van andere enzymen een belangrijke rol spelen bij de interactie tussen fosfolipiden en cytochroom P450. Bovendien is het effect van de lengte en de verzadigingsgraad van de vetzuurstaarten op de K_d alleen onderzocht voor PC. Het blijft natuurlijk de vraag of bij andere kopgroepen eenzelfde (on)afhankelijkheid van de lengte en verzadigingsgraad van de vetzuurstaarten gevonden wordt. Verder onderzoek van dit laatste aspect wordt echter belemmerd door het feit dat series van zuivere vormen van PE, PS en PI niet commercieel verkrijgbaar zijn.

In **hoofdstuk 5** werd het bestaan van specifieke fosfolipide - eiwit interacties van NADPH-cytochroom reductase onderzocht. Vergeleken met cytochroom P450 is er weinig tot geen aandacht besteed aan interacties tussen fosfolipiden en NADPH-cytochroom reductase en de mogelijke gevolgen daarvan. Toch is NADPH-cytochroom reductase een onmisbare component van het enzym systeem. Het stimulerend effect van fosfolipiden op de activiteit van cytochroom P450 afhankelijke reacties zou - ten dele - ook een resultaat kunnen zijn van een effect op NADPH-cytochroom reductase, waardoor dit eiwit bijvoorbeeld efficiënter zijn elektronen zou kunnen overdragen aan cytochroom P450.

Op basis van de resultaten van ^{31}P-NMR experimenten en chemische analyse werd geconcludeerd dat NADPH-cytochroom reductase een voorkeur heeft voor de negatief geladen fosfolipiden fosfatidylserine (PS) en fosfatidylinositol (PI). Bovendien bleek uit experimenten naar mogelijke consequenties van een speciale interactie van NADPH-cytochroom reductase met PS en PI dat (1) PS en PI een significant ander effect hadden op de quenching van de tryptofaan fluorescentie door DPH-PC dan PE en PC en dat (2) de V_{max} van cytochroom P450 IIB1 afhankelijke O-dealkylering van pentoxyresorufine in aanwezigheid van 1:1 mengsels van PS : PC en PI : PC respectievelijk hoger en lager waren in vergelijking tot de V_{max} bij een 1:1 mengsel van PE : PC of PC alleen. Deze waarnemingen zouden het best te verklaren zijn door een PS en PI specifieke verandering in de conformatie van NADPH-cytochroom reductase. Het feit dat de specifieke interactie in beide gevallen negatief geladen fosfolipiden betreft wijst in eerste instantie naar een mogelijke rol van de lading van het fosfolipide. Echter, het feit dat de V_{max} in aanwezigheid van PI lager is dan die in aanwezigheid van PC en PE terwijl in aanwezigheid van PS de V_{max} juist hoger is, wijst erop dat de lading in ieder geval niet de enige factor is die van belang is voor de invloed van fosfolipiden op NADPH-cytochroom reductase.

In **hoofdstuk 6** wordt de redox cycling van alkoxyresorufines en het produkt van hun omzetting door cytochroom P450, nl. resorufine, door NADPH-cytochroom reductase bestudeerd. Redox cycling is een proces waarbij een substraat door, in dit geval, NADPH-cytochroom reductase 1-elektron gereduceerd wordt. Dit elektron wordt op zijn beurt weer overgedragen aan moleculair zuurstof en het substraat komt weer in zijn uitgangsvorm terug en kan aan een nieuwe cyclus deelnemen. Tijdens dit proces worden reactieve zuurstof tussenprodukten gevormd die lipidperoxidatie en / of cytochroom P450 inactivatie kunnen veroorzaken. Met name in systemen waarin de NADPH-cytochroom reductase concentratie relatief hoog is kan dit proces een storende factor worden door het verbruik van reductie equivalenten en de mogelijke inactivatie van eiwitten. In de gereconstitueerde systemen die in dit proefschrift gebruikt zijn kan dit proces een belangrijke rol spelen omdat de verhouding NADPH-cytochroom reductase : cytochroom P450 in deze systemen zo'n 15 tot 30 maal hoger dan in de in vivo situatie. Daar komt

nog bij dat cytochroom P450 gevoelig is voor lipidhydroperoxides - gevormd tijdens lipidperoxidatie - en reactieve zuurstof species.

Uit de resultaten blijkt dat bij fysiologische pH alkoxyresorufines veel betere substraten zijn voor redox cycling dan resorufine. Het onvermogen van resorufine om redox cycling te stimuleren bleek te liggen in het feit dat bij fysiologische pH resorufine hoofdzakelijk in zijn gedeprotoneerde vorm voorkomt en dat deze vorm veel minder dan de geprotoneerde vorm in staat is redox cycling te ondergaan. Met behulp van AM1 molecuul orbitaal computer berekeningen werd aangetoond dat de energie (E) van de laagste niet gevulde orbitaal (LUMO = de energie van de orbitaal waarin het elektron wordt geplaatst tijden redox cycling) van de gedeprotoneerde vorm hoger is dan de E_{LUMO} van de geprotoneerde vorm van resorufine. Tevens bleek 1-elektron reductie van de geprotoneerde vorm energetisch de voorkeur te hebben boven de 1-elektron reductie van de gedeprotoneerde vorm met 363.5 kJ/mol. Aanvullende computerberekeningen toonden aan dat het 1-elektron gereduceerde resorufine wordt geprotoneerd op de zuurstof van de intramoleculaire semichinonimine groep voordat het wordt gereduceerd met een tweede elektron. Tot slot bleek dat incorporatie van NADPH-cytochroom reductase in een kunstmatige membraan een positief effect had op de redox cycling van resorufine. Dit werd verklaard door een stijging in de concentratie van de geprotoneerde vorm in het membraan door (1) partitie van de geprotoneerde vorm in het membraan en / of (2) een membraan geïnduceerde verschuiving in het protoneringsevenwicht van resorufine in het voordeel van de geprotoneerde vorm. Dit resultaat wijst op een rol van de membraan in het concentreren van de apolaire substraten van het cytochroom P450 : NADPH-cytochroom reductase systeem [8-10]

In **hoofdstuk 7** werd nader onderzoek gedaan naar de in hoofdstuk 7 gevonden resultaten wat betreft de voorspellende waarde van AM1 berekeningen voor de capaciteit van stoffen om redox cycling te ondergaan. Naast de redox cycling van resorufines werd gekeken naar de redox cycling van chinonen. Chinonen zijn toxische stoffen die veel gebruikt worden in de chemie. Daarnaast worden ze ook als medicijn gebruikt in de strijd tegen kanker vanwege hun toxische karakter. De toxische werking van chinonen wordt toegeschreven aan hun capaciteit om te binden aan cellulaire nucleofiele macromoleculen en/of hun

vermogen om redox cycling te stimuleren. Van 1,4-benzochinon is echter bekend dat het een slecht substraat is voor redox cycling. In de literatuur wordt als oorzaak voor de slechte redox cycling capaciteit een erg hoge 1-elektron reductie potentiaal gegeven. Uit de resultaten van hoofdstuk 7 blijkt echter dat bij fysiologische pH 1,4-benzochinon gemakkelijk een twee elektron reductie door NADPH-cytochroom reductase ondergaat resulterend in de vorming van 1,4-hydrochinon. In plaats van zijn elektron over te dragen aan moleculair zuurstof wordt het 1-elektron gereduceerde semichinon geprotoneerd en vervolgens gereduceerd door een tweede elektron. Wanneer de pH echter wordt verhoogd tot boven 9 blijkt 1,4-benzochinon wel in staat tot redox cycling. Tevens bleek er een overeenkomst te bestaan tussen de pH en concentratie afhankelijkheid van de redox cycling in een systeem met NADPH-cytochroom reductase en 1,4-benzochinon ofwel 1,4-hydrochinon. Op basis van deze waarnemingen werd geconcludeerd dat 1,4-benzochinon in staat is om redox cycling te katalyseren via zijn gedeprotoneerde, twee-elektron gereduceerde vorm; zij het alleen bij relatief hoge pH, waarbij de gedeprotoneerde vorm voldoende aanwezig is. De resultaten van hoofdstuk 6 en 7 wijzen op het belang van het protoneringsevenwicht van de 1- en 2-electron gereduceerde vormen in het redox cycling proces.

In de bovenstaande alinea's zijn in het kort de resultaten weergegeven van het onderzoek naar de rol van de fosfolipiden in het cytochroom P450 : NADPH-cytochroom reductase systeem zoals dat in het kader van dit proefschrift is uitgevoerd. Sinds 1968 [11] is de rol van fosfolipiden in dit enzymsysteem onderwerp geweest van menige studie waarin veel vragen werden beantwoord maar evenzoveel nieuwe vragen opgeroepen. Dit proefschrift levert niet de antwoorden op deze nieuwe vragen. Daarvoor is het cytochroom P450 enzym systeem te complex en zijn de diversiteit van de fosfolipiden en de effecten die ze induceren te groot. De bedoeling van dit onderzoek was een aantal aspecten van fosfolipiden en het cytochroom P450 systeem waaraan tot nu toch nog maar weinig aandacht is besteed verder uit te diepen en op deze manier de kennis (en het begrip) hierover te vergroten.

De resultaten van de experimenten beschrijven nieuwe effecten van fosfolipiden op het enzym systeem. Daarnaast wordt er ook aanvullend bewijs geleverd voor sommige van de reeds bestaande hypothesen die in hoofdstuk 2 staan vermeld. De conclusies omtrent de rol van

het membraan(fosfolipide) in het cytochroom P-450 enzymsysteem, zoals ze zijn gevonden in dit proefschrift, worden hieronder nog eens puntsgewijs samengevat.

1) Cytochroom P450 enzymen kunnen verschillen in de manier waarop ze ingebouwd zijn in het membraan waardoor ze een verschillende gevoeligheid kunnen vertonen voor cumene hydroperoxide.

2) De manier van incorporatie van cytochroom P450 enzymen in microsomale en gereconstitueerde systemen is vergelijkbaar.

3) Fosfolipiden kunnen de kinetische parameters K_m en V_{max} van cytochroom P450 afhankelijke reacties in gereconstitueerde systemen beïnvloeden.

4) De uitkomst van de invloed van fosfolipiden op de kinetische parameters van cytochroom P450 afhankelijke reacties is niet hetzelfde voor alle cytochroom P450 enzymen.

5) De affiniteit van cytochroom P450 voor NADPH-cytochroom reductase in een gereconstitueerd systeem is afhankelijk van de vetzuursamenstelling van het fosfolipide molecuul dat bij reconstitutie wordt toegevoegd

6) Fosfolipiden kunnen de K_d van cytochroom P450 voor het substraat verlagen.

7) De K_d van cytochroom P450 enzymen voor fosfolipiden is afhankelijk van de kopgroep van het fosfolipide molecuul en de verzadigingsgraad van de vetzuurstaarten maar onafhankelijk van de lengte van de acylketen.

8) Er bestaat een speciale interactie tussen NADPH-cytochroom reductase en de negatief geladen fosfolipiden PS en PI.

9) Het membraan fungeert als een plaats waar apolaire substraten zich kunnen ophopen.

10) Het membraan zorgt voor een verschuiving in het overall protoneringsevenwicht van het substraat naar de geprotoneerde kant.

Naast deze conclusies zijn er ook nog een aantal andere interessante waarnemingen gedaan die geen betrekking hebben op de rol van fosfolipiden in het cytochroom P450 enzym systeem maar die ook de moeite van het vermelden waard zijn.

(1) De parameters die de manier bepalen op welke wijze cytochroom P450 enzymen ingebouwd worden in het membraan worden niet beinvloedt door de zuiveringsprocedure.

(2) AM1 computer berekeningen zijn zeer bruikbaar bij het onderzoek naar redox cycling capaciteit van chemicaliën.
(3) De protoneringsevenwichten van 1- en 2- electron gereduceerde stoffen is van belang voor hun vermogen tot redox cycling.

Tot slot, gezien de resultaten van de experimenten in dit proefschrift moet hier misschien nog een belangrijke conclusie aan toegevoegd worden. Het effect van fosfolipiden in het cytochroom P450 enzymsysteem blijkt afhankelijk te zijn van veel factoren zoals het soort cytochroom P450 enzym, de vetzuurstaarten en kopgroep van het fosfolipide, de P450 : reductase verhouding, de fosfolipide : P450 verhouding en het soort gereconstitueerde systeem dat gebruikt is. Daarom is, voor een goed begrip van het mechanisme van fosfolipide stimulatie van cytochroom P450-afhankelijke reacties, een gedetaileerd onderzoek naar het effect van alle (mengsels van) fosfolipiden op alle cytochroom P450 enzymen bij verschillende P450 : reductase en fosfolipide : P450 verhoudingen noodzakelijk. Dit vraagt een hoop tijd hoewel sommige zaken al (ten dele) zijn onderzocht in de afgelopen 25 jaar. In veel gevallen zijn deze resultaten echter niet vergelijkbaar doordat de experimenten onder steeds verschillende condities hebben plaatsgevonden. Het is daarom aan te raden in de toekomst, net als voor de nomenclatuur van cytochroom P450, te komen tot gestandaardiseerde condities voor experimenten met gereconstitueerde systemen zodat de resultaten van verschillende laboratoria in de toekomst gemakkelijker vergeleken kunnen worden.

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Chapter 10

Uitleg voor niet-ingewijden

Omzetting van stoffen in het lichaam

Gedurende het hele leven komt de mens in aanraking met tal van lichaamsvreemde stoffen van natuurlijke en niet-natuurlijke oorsprong. Deze lichaamsvreemde stoffen worden ook wel *xenobiotica* genoemd (xenos = vreemd, bios = leven). De xenobiotica kunnen het lichaam binnendringen via de natuurlijke weg zoals het maag-darm kanaal, de huid en de luchtwegen maar ook via kunstmatige wegen zoals via injecties of een infuus. Om eventuele giftige werkingen tot een minimum te beperken, moeten deze stoffen zo snel mogelijk het lichaam weer verlaten. Door het in veel gevallen vetachtige karakter van deze stoffen lossen ze echter slecht op in water en hebben ze de neiging zich op te hopen in het lichaam (met name in het vetweefsel) in plaats van uitgescheiden te worden via de urine of ontlasting. Gelukkig beschikt de mens, maar ook dieren, bacteriën, planten enz. over een machinerie die in staat is de chemische structuur en daarmee het karakter van deze stoffen dusdanig te veranderen dat ze beter oplosbaar worden in water. Dit proces heet *biotransformatie* . Het proces van biotransformatie verloopt in twee fasen. In de eerste fase wordt er iets aan de structuur van het xenobioticum veranderd (*modificatie*). Er wordt als het ware een "haakje" gecreëerd waaraan in de tweede fase een goed water-oplosbare groep kan worden "opgehangen" (*conjugatie*).

Een belangrijk eiwit dat fase I reacties katalyseert is het cytochroom P450. Dit eiwit maakt met een aantal andere eiwitten, waarvan NADPH-cytochroom reductase de belangrijkste is, deel uit van het cytochroom P450 enzymsysteem. Dit enzymsysteem kan een lichaamsvreemde stof op tal van manieren omzetten. Over h*et al.*gemeen leidt dit tot een ontgifting (*detoxificering*) van het xenobioticum, doch in sommige gevallen kan het ook resulteren in de vorming van een omzettingsprodukt dat giftiger is dan de uitgangsstof (*bioactivering*). Het moge duidelijk zijn dat de verhouding van detoxificering en bioactivering, alsmede de snelheid waarmee het xenobioticum wordt omgezet van groot belang is voor de uiteindelijke giftigheid (*toxiciteit*). Van het belangrijkste eiwit van het cytochroom P450 enzymsysteem, het cytochroom P450 eiwit zelf, bestaan verschillende vormen. Deze verschillende vormen van cytochroom P450 onderscheiden zich van elkaar door verschillen in de structuur van het eiwit. Door deze structurele verschillen zijn deze cytochroom P450 vormen in staat om verschillende groepen van stoffen (*substraten*) om te zetten (te

metaboliseren). Dit is het beste duidelijk te maken met behulp van het "sleutel en slot" principe. De vorm van het xenobioticum, de "sleutel", moet overeenkomen met de vorm van het "slot", het cytochroom P450 eiwit, anders kan het xenobioticum niet omgezet worden. Een andere structuur van het cytochroom P450 betekent een ander "slot" waarop andere "sleutels" (substraten) passen.

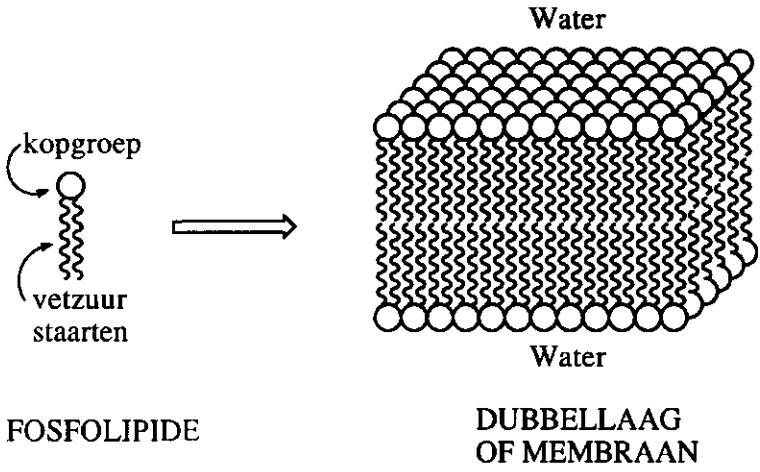
Het cytochroom P450 enzymsysteem kan een grote verscheidenheid van reacties katalyseren. Van meer dan 250.000 xenobiotica is het tot nu toe bekend dat ze door cytochroom P450 omgezet worden. De meeste van deze reacties zijn zogenaamde monooxygenase reacties wat betekent dat een zuurstof molecuul, bestaande uit twee zuurstof atomen, wordt gesplitst waarbij één zuurstof atoom wordt ingebouwd in het substraat en het andere zuurstof atoom wordt ingebouwd in een water molecuul. Het in het xenobioticum ingebouwde zuurstof atoom is in veel gevallen het "haakje" voor de fase II reactie.

Het membraan

De omzetting van lichaamsvreemde stoffen bij zoogdieren vindt voornamelijk plaats in de lever. De lever is net als de rest van ons lichaam opgebouwd uit cellen. Iedere cel wordt omgeven door een soort "velletje", het membraan, die het inwendige van de cel afschermt van het externe milieu. Een membraan is voor een groot gedeelte opgebouwd uit zogenaamde *fosfolipiden*. De structuur van deze fosfolipiden heeft een tweeledig karakter; ze zijn opgebouwd uit een stuk dat goed in water oplost, de kopgroep, en een stuk dat slecht oplost in water, de vetzuurstaarten (Figuur 1). Fosfolipiden kunnen verschillende kopgroepen bezitten waarvan choline, ethanolamine, serine en inositol de meest belangrijke zijn. De fosfolipiden worden vaak ingedeeld op basis van het soort kopgroep dat het fosfolipide bevat. Ook de vetzuurstaarten van het fosfolipide molecuul zijn variabel. Doordat er twee vetzuren aan een fosfolipide molecuul kunnen zitten en doordat er keuze is uit veel verschillende vetzuren is de variatie in de vetzuurstaarten veel groter dan in de kopgroepen.

Het menselijk lichaam bestaat voor ongeveer 80% uit water en in dit waterige milieu oriënteren de fosfolipiden zich dusdanig dat de vetzuurstaarten zich naar elkaar toewenden en alleen de kopgroepen, die goed oplossen in water, in contact komen met de waterige oplossing. In

de praktijk komt dit erop neer dat de fosfolipiden een dubbellaag of membraan vormen, waarbij het inwendige van deze dubbellaag gevormd wordt door de vetzuurstaarten en de buitenkant door de kopgroepen van de fosfolipiden (Figuur 10.1).



Figuur 10.1: Structuur van fosfolipide en membraan

In de levercel bevinden zich een groot aantal cel organellen die elk op hun beurt ook weer omgeven zijn door zo'n membraan. Het cytochroom P450 enzymsysteem is vooral gebonden aan het membraan van één van deze cel organellen, namelijk het *endoplasmatisch reticulum*. Het membraan en met name de fosfolipiden, blijken van invloed te zijn op de werking van het cytochroom P450 enzymsysteem.

Testsystemen

Het onderzoek naar de omzetting van lichaamsvreemde stoffen kan om ethische redenen alleen in zeer speciale gevallen bij mensen worden verricht. Daarom wordt er gebruik gemaakt van proefdieren. In veel gevallen zijn dit ratten. Het onderzoek kan gebruik maken van verschillende testsystemen. Bij zogenaamd *in vivo* onderzoek wordt gebruik gemaakt van de dieren als zodanig. De lichaamsvreemde stof wordt bijvoorbeeld via het drinkwater toegediend en de urine, ontlasting en / of het bloed van het proefdier wordt opgevangen en geanalyseerd om de omzettingsprodukten te karakteriseren. Dit soort systemen worden veel gebruikt bij toxicologisch onderzoek. *In vivo* onderzoek is vaak zeer lastig, kostbaar en belastend voor het proefdier. Daarom wordt vaak bij

voorkeur gebruik gemaakt van zogenaamde *in vitro* testsystemen ("in de reageerbuis"). Bij dit soort, meer artificiele systemen wordt gebruik gemaakt van uit-ratten geïsoleerde levers. Het voordeel van *in vitro* systemen is dat er vele honderden of zelfs duizenden bepalingen uitgevoerd kunnen worden met materiaal van 1 proefdier tegenover slechts 1 bij *in vivo* onderzoek. *In vitro* systemen zijn dus proefdier besparend. Het experimentele materiaal dat uit de levers verkregen kan worden, kan uiteenlopen van geïsoleerde levercellen (*hepatocyten*), membranen (*microsomen*) tot zelfs gezuiverde eiwitten / enzymen. Van de verschillende *in vitro* systemen staat het systeem met de geïsoleerde levercellen het dichtst bij de originele situatie en wordt daarom *net al.s* het *in vivo* systeem vaak in toxicologisch onderzoek gebruikt. Het verkrijgen van levercellen is echter nogal bewerkelijk en de houdbaarheid van de levercellen is beperkt.

Voor het bestuderen van het eigenlijke proces van de omzetting van een lichaamsvreemde stof door cytochroom P450 en de factoren die dit proces beïnvloeden kunnen in veel gevallen de andere *in vitro* testsystemen gebruikt worden. Het meest gebruikt is het systeem met microsomen. Dit zijn stukjes van de membraan van het *endoplasmatisch reticulum* die ontstaan via het malen van rattelever materiaal. Met behulp van ultracentrifuge kunnen de microsomen van ander lever weefsel gescheiden kunnen. In deze membraanstukjes is het cytochroom P450 enzymsysteem ingebed. Bij andere *in vitro* testsystemen wordt gebruik gemaakt van de individuele eiwit componenten van het enzymsysteem die gezuiverd worden uit rattelevers. Het cytochroom P450 enzymsysteem wordt dan weer in elkaar gezet (*reconstitutie*) door de eiwit componenten en fosfolipiden opnieuw bij elkaar te brengen. Dit testsysteem heeft het voordeel dat het enzymsysteem naar wens samengesteld kan worden en dat storende factoren tot een minimum beperkt kunnen worden. Een nadeel is echter dat dit systeem ver verwijderd is van de oorspronkelijke situatie in de levercel. Er zijn twee verschillende gereconstitueerde systemen te onderscheiden: (i) een gereconstitueerd systeem waarin de individuele eiwitcomponenten en de fosfolipiden in oplossing blijven en (ii) een gereconstitueerd systeem waarin de eiwitcomponenten zijn ingebouwd in een kunstmatige fosfolipide membraan.

In dit proefschrift is met name gebruik gemaakt van microsomen en van de twee gereconstitueerde testsystemen omdat de mechanistische

invloeden van fosfolipiden op het cytochroom P450 enzymsysteem onderzocht werden. Voor dit soort onderzoek is het wenselijk dat de componenten van het enzymsysteem systematisch en gemakkelijk gewijzigd en beïnvloedt moeten kunnen worden.

Doel van dit onderzoek

Een volledig begrip van de werking van het cytochroom P450 enzymsysteem is zeer belangrijk voor de toxicologie ("leer der vergiften") bij het vaststellen van de bioactivering en detoxificering van een lichaamsvreemde stof. Maar ook voor de geneeskunde en de farmacologie ("leer der geneesmiddelen") is een goede kennis van dit enzymsysteem van groot belang, met name omdat de werking van het P450 enzymsysteem mede bepalend is voor de verblijftijd van geneesmiddelen en zijn metabolieten in het lichaam en derhalve voor de benodigde dosering. Op het ogenblik wordt het cytochroom P450 enzymsysteem al gebruikt in de farmaceutische industrie bij het screenen van potentiële geneesmiddelen. Ook in de organische chemie zijn er toepassingen beschreven voor het cytochroom P450 enzymsysteem, met name bij de stereo- en regioselectieve synthese van chemicaliën. Daarnaast zou het ook nog gebruikt kunnen worden in de strijd tegen de steeds maar toenemende milieuverontreiniging voor het verwijderen van schadelijke chemicaliën.

De afgelopen 20 jaar is onderzoek gedaan naar de rol van de fosfolipiden in het cytochroom P450 enzymsysteem. Dit heeft echter nog altijd niet geleid tot een eensluidende conclusie over de rol en het belang van fosfolipiden voor het systeem mede als gevolg van de complexiteit van het enzymsysteem en de grote verscheidenheid aan effecten die door fosfolipiden geïnduceerd worden. Het doel van het onderzoek is dan ook om verder inzicht te verkrijgen in de rol van de membraan en membraanfosfolipiden bij het functioneren van het cytochroom P450 enzymsysteem. Hierbij wordt niet alleen gekeken naar het cytochroom P450 eiwit zelf maar ook naar het NADPH-cytochroom reductase.

Dit proefschrift

In de eerste twee hoofdstukken van dit proefschrift worden in het kort de structurele en katalytische eigenschappen van het cytochroom P450 enzymsysteem beschreven (*Hoofdstuk 1A*) alsmede de structurele aspecten van fosfolipiden en de membraan van het endoplasmatisch

reticulum en hypotheses voor het stimulerende effect van fosfolipiden op het enzymsysteem (*Hoofdstuk 1B*).

In de hoofdstukken 2 tot en met 7 worden de resultaten besproken van de experimenten zoals die de afgelopen vier jaar zijn uitgevoerd. De eerste hoofdstukken (*Hoofdstuk 2, 3 en 4*) hebben betrekking op de relatie cytochroom P450 - fosfolipiden, *Hoofdstuk 5* heeft betrekking op de relatie NADPH-cytochroom reductase - fosfolipiden en de *Hoofdstukken 6 en 7* gaan over een neven reactie die tijdens cytochroom P450 afhankelijke reacties kan optreden; de NADPH-cytochroom reductase afhankelijke redox cycling. Het proefschrift wordt afgesloten met een hoofdstuk met samenvattingen, evaluaties van de resultaten en conclusies (*Hoofdstuk 8*).

Curriculum Vitae

Walter Balvers werd op 4 juni 1964 geboren in Utrecht. In 1982 behaalde hij zijn VWO-diploma aan het Alberdingk Thijm College in Hilversum. In datzelfde jaar begon hij ook met de studie Scheikunde aan de Rijksuniversiteit Utrecht. In januari 1984 behaalde hij zijn propadeutisch examen. De doctoraalfase werd doorgebracht bij de vakgroep Biochemie bij de groep van prof.dr. K.W.A Wirtz waar onder leiding van dr. B. Roelofsen onderzoek werd verricht naar afwijkingen in de membraan van de rode bloedcel van patiënten die lijden aan de ziekte van Batten-Spielmeyer-Vogt. Na het voltooien van de doctoraalscriptie met de titel "Ca^{2+}-geïnduceerde echinocytose van de erythrocyt; de rol van de membraan" werd in oktober 1987 het doctoraal scheikunde (nieuwe stijl) behaald.

In oktober 1988 trad hij in dienst van de Landbouwuniversiteit in Wageningen als assistent in opleiding bij de vakgroep Biochemie. Daar heeft hij het in dit proefschrift beschreven onderzoek uitgevoerd onder leiding van prof.dr. C.Veeger en dr.ir. I.M.C.M. Rietjens.