STUDIES ON NATIVE AND RECONSTITUTED FMN-DEPLETED NADPH-CYTOCHROME P-450 REDUCTASE

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STELLINGEN

1. Het voorkomen van veel verschillende wijzen van nomenclatuur bij reeds lang bekende iso-enzymen is niet alleen verwarrend voor de betrokken onderzoekers, maar doet ook vermoeden dat andere dan zuiver wetenschappelijke belangen een rol spelen.
   - W.B. Jacoby, B. Ketterer & B. Mannervik, Bioch.Pharm. 33, (1984), 2539-2540.
   - T.J. Mantle in Glutathione S-Transferases and Carcinogenesis (1987), ed. T.J. Mantle, C.B. Pickett & J.D. Hayes, Taylor & Francis London, New York and Philadelphia.

2. De door Wolfes en Pingoud verkregen resultaten met betrekking tot site directed mutagenesis aan Eco R1 zijn niet volledig in overeenstemming met de X-ray resultaten van dit restrictie-enzym van McClarin en Frederick en kunnen scherper geformuleerd worden.
   - H. Wolfes, A. Pingoud et al., Nucl.Acid.Res. 14, 22 (1986), 9063.
   - J. McClarin, C. Frederick, J.M; Rozenberg et al., Science 234 (1986), 1526.

3. De activiteitsbepalingen m.b.v. het gebruik van artificiële i.p.v. natieve electronen acceptoren verdient zeker in het geval van NADPH-cytochroom P-450 reductase meer aandacht.
   - dit proefschrift.

4. Het feit dat alcohol een bepaalde vorm van cyt P-450 induceert (cyt P-450-alc) en dat deze vorm ook procarcinogenen kan omzetten wijst op toenemende kans op kanker bij alcoholgebruik.
   - J.M. Lasker, J. Rancy, S. Kubota, B.P. Bloswick, M. Black & C.S. Lieber, Biochm.Biophys.Res.Commun. 148 (1987), 232-238.

5. In tegenstelling tot wat Schreuder et al. beweren speelt de C(4) carbonylgroep in het catalytisch mechanisme van PHBH nauwelijks een rol.
   - H. Schreuder, W. Hol en J. Drenth In Flavins and Flavoproteins (1987) eds. D.B. McCormick & D.E. Edmondson, W. de Gruyter Berlin, in press.

6. De biotechnologische produktie van geneesmiddelen en pesticiden moet kunnen resulteren in een verbod op het zonder meer gebruik van racemische mengsels van deze verbindingen.
   - Ariens, Science 7, (1986), 200-205.

7. Het door Nisimoto geleverde bewijs dat FAD stevig gebonden is aan FAD-vrij NADPH-cytochroom P-450 reductase wordt niet ondersteund door de resultaten beschreven in hoofdstuk 3 van dit proefschrift.
   - Y. Nisimoto, J.Biol.Chem. 259, (1984), 2480-2483.
   - Hoofdstuk 3 van dit proefschrift.

8. Gezien het grote aantal werkzoekenden is het bedroevend om te zien (horen) hoe er in het algemeen met de belangen van sollicitanten wordt omgesprongen.

9. Specialisten zijn mensen die steeds meer van steeds minder weten.

10. Het is onwaarschijnlijk dat voor het jaar 2000 2D-NMR-spectra, van zelfs eenvoudige eiwitten (meer dan 100 aminozuren), volledig geautomatiseerd geanalyseerd zullen worden. Het blijft voorlopig monnikkenwerk.

Studies on native and reconstituted FMN-depleted NADPH-cytochrome P-450 reductase

Peter Bonants
Wageningen, 18 december 1987

PETER BONANTS

STUDIES ON NATIVE AND RECONSTITUTED FMN-DEPLETED NADPH-CYTOCHROME P-450 REDUCTASE

Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 18 december 1987
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

Aan mijn ouders

Aan Nica

VOORWOORD

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CONTENTS

LIST OF ABBREVIATIONS

2'5'ADP : Adenosine 2'5'-diphosphate

2'AMP : Adenosine 2'-monophosphate

APO : FMN-depleted NADPH-cytochrome P-450 reductase

CD : Circular Dichroism

cDNA : copy DNA

DEAE : Diethyl amino ethyl

DTNB : 5,5'-dithio-bis(2-nitrobenzoate)

DTT : Dithiothreitol

EDTA : Ethylene diamine tetra acetic acid

EPR : Electron Paramagnetic Resonance

FAD : Flavin Adenine Dinucleotide

FMN : Flavin Mono Nucleotide

kDa : kilo Dalton

k_{ps} : pseudo first order rate constant

λ_{exc} : excitation wavelength

mRNA : messenger RNA

NADPH : Nicotinamide Adenine Dinucleotide Phosphate, reduced form

$NADP^+$: Nicotinamide Adenine Dinucleotide Phosphate, oxidized form

nm : nanometer

nmole : nanomole

NMR : Nuclear Magnetic Resonance

ns : nanosecond

OX : Oxidized

PAGE : Polyacrylamide gelelectrophoresis

PC : Phosphatidylcholine

PCMB : p-hydroxychloromercuri benzoate

PMSF : Phenyl methane sulphonyl fluoride

PPM : parts per million

REC : FMN reconstituted FMN-depleted NADPH-cytochrome P-450 reductase

SDS : Sodium dodecyl sulphate

SQ : Semiquinone (one-electron reduced)

TLC : Thin-layer chromatography

TNBS : Sodium 2,4,6-tri-nitrobezene sulfonate

UC : Ultracentrifugation

UV : Ultraviolet

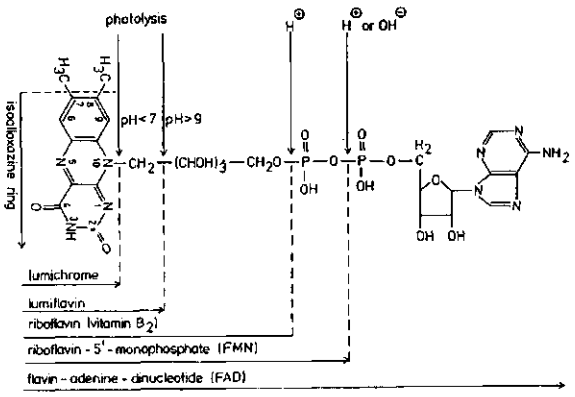


FIGURE 1
Structures of some common flavins

CHAPTER 1

INTRODUCTION

In nature there are an enormous number of enzymes active in various species. This thesis deals with one of those enzymes , namely NADPH-cytochrome P-450 reductase. This enzyme is part of a group of enzymes called flavoproteins , which contain one or more flavins. The flavin is a prosthetic group which is covalently or non-covalently bound to enzymes and has a specific function in the overall activity of the enzyme in question. Fig. 1 shows the most common flavin cofactors which are derived from vitamine B_2. NADPH-cytochrome P-450 reductase is present particularly in mammalian liver microsomes . It is an unusual membrane-bound flavoprotein because it contains two different flavins : FAD (Flavin Adenine Dinucleotide) and FMN (Flavin Mono Nucleotide) in one polypeptide chain.

History:

The first report on this enzyme was made by Horecker in 1949 [1]. He reported on an NADPH-mediated cytochrome c reductase in animal tissue which contained only one flavin (FAD). Despite the excellent work, the physiological role of the enzyme and the localization of the enzyme in the tissue remained unclear. However at that time one could not foresee a possible function for this flavoprotein in microsomal electron trans-

port. In the mid 1950's Strittmatter and Velick [2] observed in their studies a different microsomal fraction which reduced cytochrome c 20 times faster in the presence of NADPH rather with NADH as electron donor. They suggested that this activity might be the same as that described by Horecker. In 1962 Williams and Kamin [3], and shortly thereafter Phillips and Langdon [4], showed that NADPH-cytochrome c reductase was localized in the microsomes. Both groups speculated on the possible role of this flavoprotein in aromatic and steroid hydroxylations , and drug demethylations . Phillips and Langdon [4] presented some evidence for the participation of this enzyme in a microsomal electron transport system not involving cytochrome c. In the mean time Williams [5] made the discovery of a carbon monoxide-binding pigment in rat liver microsomes exhibiting an intense absorption at 450 nm in its reduced carbon monoxide bound form . Omura and Sato [6] proved in 1962 that this protein contains heme and studied the heme protein in more detail. In 1963 and 1964 it was shown by Estabrook [7] and Cooper [8] that this cytochrome P-450 was involved in a great variety of hydroxylation and oxidative dealkylation reactions. The selective increase of both cytochrome P-450 and NADPH-cytochrome P-450 reductase in microsomes from drug-treated animals was a first indication that the reductase was also involved in drug and steroid metabolism [9-12]. The final evidence for its participation in drug metabolism came from immunochemical and reconstitution experiments. Kuriyama [13] blocked the cytochrome c reduction by a specific antibody and Omura [14] reported the inhibition of a microsomal xenobiotic hydroxylation reaction by this specific antibody against reductase, other reactions followed [15-18]. Reconstitution of a cytochrome P-450 monooxygenase system was first reported by Lu and Coon [19] who introduced a new purification procedure for the protein.

Isolation and purification:

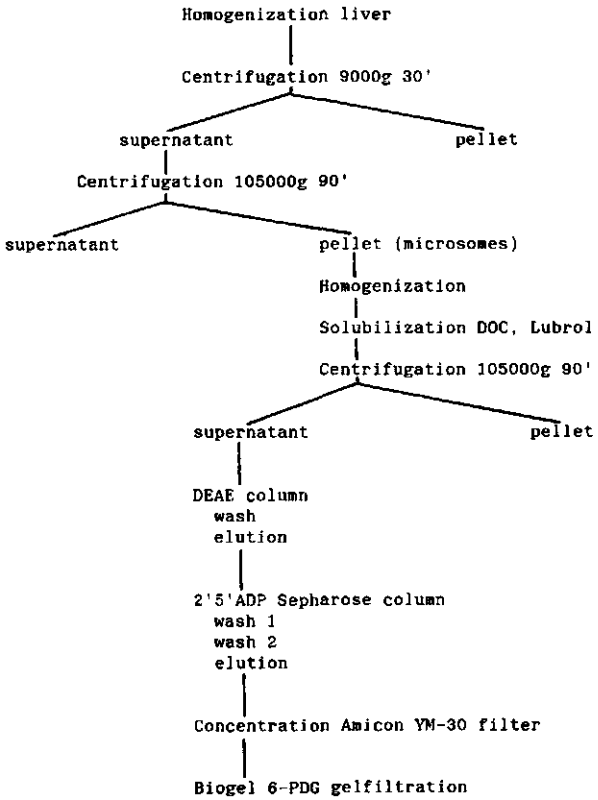
The isolation procedure of NADPH-cytochrome P-450 reductase has made an enormous development since its discovery. Horecker described the purification procedure of the reductase from an acetone powder of whole pig liver from which the enzyme was released by trypsin digestion [1]. Masters [20] used the pancreatic steapsin procedure of Williams and Kamin [3] on pig liver microsomes followed by purification steps including pH precipitation, ammonium sulphate fractionation, hydroxylapatite chroma-

tography and calcium gel adsorption. The earliest report to solubilize the NADPH-cytochrome P-450 reductase from liver microsomes came from Coon [19] followed soon by others (Dignam and Strobel [21,22], Yasukochi and Masters [23], Vermillion and Coon [24,25]). They all obtained a homogeneous preparation of the reductase which could be reconstituted with cytochrome P-450 and phosphatidylcholine to an active drug metabolism system. The earlier preparations were not reconstitutable.

The introduction of a biospecific affinity chromatography step in the isolation procedure was made by Yasukochi and Masters in 1976 [23]. This method takes advantage of the high affinity of the 2'-phosphate group of NADPH for the enzyme and the slightly lower binding constant of the reductase for 2' AMP than for $NADP^+$, which serves as an excellent eluting substrate after binding of the reductase to 2'5'-ADP-Sepharose 4B (Sepharose 4B-bound N(6)-(6-aminohexyl)-adeno-sine 2'5'-biphosphate).

The isolation procedure used in this thesis is summarized (Fig.2).

FIGURE 2
Isolationprocedure at 4°C



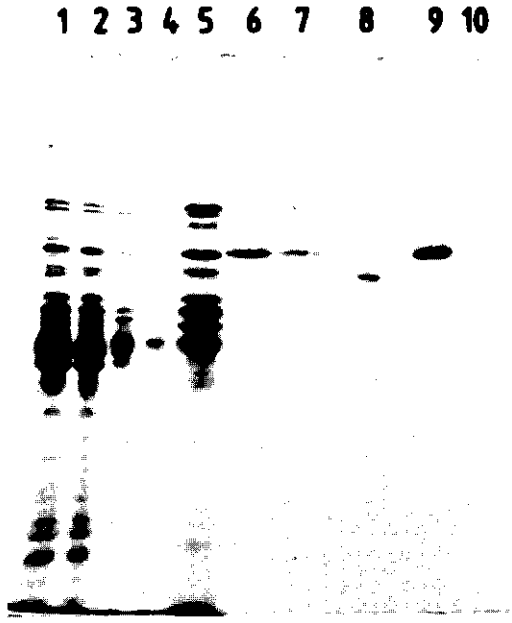


FIGURE 3
Photo of SDS gel with samples at different steps of the purification
1: microsomes; 2: solubilized microsomes; 3,4: minor DEAE-pool;
5: main DEAE pool; 6,7,9: 2'5'ADP pool; 8: markers (67 and 43 kDa);
10: FMN-depleted reductase.

A photograph of an SDS gel, showing samples from different steps of the isolation procedure, is shown in Fig. 3.

Molecular weight:

The purified reductase from different organs and from different species such as rat , rabbit and pig resulted in a homogeneous preparation with a molecular weight of 78 kDa [23,25-30]. The molecular weight of this sample (78.000 Da) differed from the one reported previously by Masters (68.000 Da,[31]) and it revealed that the isolation procedure was responsible for the difference in molecular weight. The smaller reductase was derived from the larger one by proteolysis with trypsine [25]. Reductase preparations solubilized by hydrolytic methods are unable to support the cytochrome P-450 catalyzed hydroxylation reactions, but are able to reduce various exogenous electron acceptors . The higher molecular weight, detergent-solubilized preparations, however, catalyzes the direct reduction of exogenous electron acceptors as well as cytochrome P-450 mediated substrate hydroxylation reactions. Evidence has been presented [32] that the 10.000 Da sequence of the reductase is responsible for conferring reactivity with cytochrome P-450 and is also

responsible for the ability of the reductase to bind to hepatic microsomes and phospholipid vesicles . Due to proteolysis of the reductase during purification some even smaller fragments of the enzyme were found on SDS gel electrophoresis (Masters, unpublished results) , this reaction is diminished in the presence of protease inhibitors .

Flavin content:

Masters and Kamin [20,33] reported that the reductase contained 2 mole of FAD/ mole of enzyme in contrast with earlier reports that stated 1 mole of flavin per enzyme (FAD). Iyanagi and Mason [34] showed unquestionable in 1973 that the reductase contained 2 flavins (FAD and FMN (Fig. 1)) in equimolar quantities. Until then all investigators found only FAD present in the enzyme. The reason why the highly fluorescent FMN was missed is that FMN binds much more less tightly than FAD. Other groups soon confirmed that their reductase preparations all contained FAD and FMN in equimolar quantities [21-24,35,36].

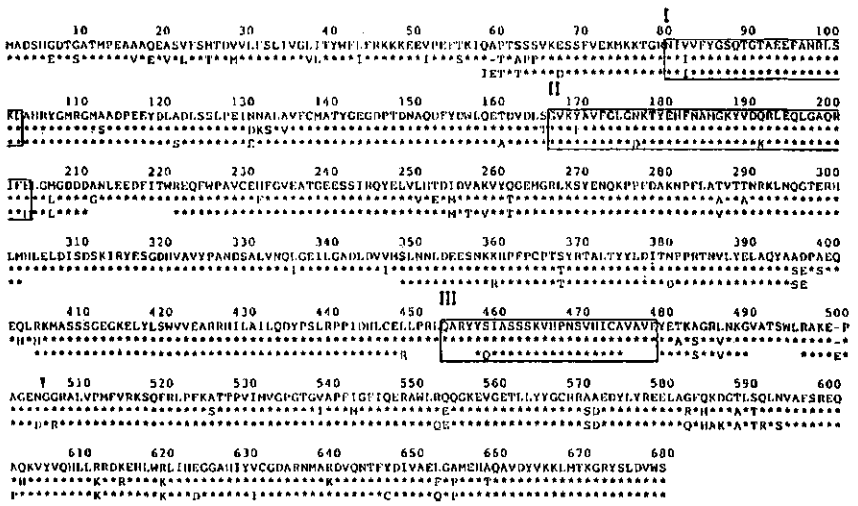


FIGURE 4
Comparison of amino acid sequences of rabbit,rat and pig NADPH-cytochrome P-450 reductase. The sequences of the rat enzyme was taken from Porter and Kasper [44] and Murakami et al. [47]. The sequence of the pig enzyme was obtained by alignment of the data reported by Vogel et al. [45] and Haniu et al. [42]. In the rat and pig sequences, asteriks indicate residues identical with those of the rabbit sequence, and letter symbols show differences from the rabbit enzyme. The dash indicates a gap inserted to optimize the alignment. The boxed sequences are segments suggested as the FMN-binding segment (Regions I and II) and the FAD-binding segment (Region III) by Porter and Kasper [44]. The vertical arrowhead indicates the site of proteolytic cleavage in pig enzyme [41]. Obtained from Katagiri et al. [46].

Amino acid analysis/sequence:

Amino acid analysis have been published on the reductase isolated from different organs and species [25,37-39]. There was much but not total homology in the amino acid content between reductases isolated from different species and from different organs. It lasted untill 1982 when the first amino acid sequence appeared in literature [40]. This was the sequence of rabbit reductase. Other investigators elucidated the sequence of reductases from other species, such as rat and pig [41-45]. The method of determining the amino acid sequence of Porter and Kasper is worth special mentioning [44]. They cloned the gene encoding for the reductase in a E. coli transformant and determined the nucleotide sequence of the mRNA for the reductase. The amino acid sequence deduced from this is in excellent agreement with that determined by direct amino acid analysis. The sequence obtained by this method agreed very well with previous sequences from other species. Also Katagiri [46] was successful in cloning of a full-length cDNA for rabbit liver NADPH-cytochrome P-450 reductase mRNA. The nucleotide sequence of this cDNA showed 85% homology to that of the rat reductase [44,47]. The amino acid sequence of rabbit reductase (one amino acid more than the one from rat) showed 91% identity with that of the rat enzyme. The segment related to binding of FMN and FAD was well conserved among rabbit, rat and pig reductases. In Fig. 4 the amino acid sequences of NADPH-cytochrome P-450 reductase isolated from these different species are summarized [46]. There is very much homology between the sequences of the three reductases. The sequence related to the AMP moiety binding site was also conserved among these species and was also found in the amino acid sequence of NADH-cytochrome b_5 reductase, another flavoenzyme in the microsomal electron transport system [48].

Oxidation-reduction properties:

The transfer of electrons from NADPH through the reductase to acceptors has been of considerable interest to investigators for many years. Much attention has been focused on the reduction and oxidation of the reductase itself. Masters and Kamin [20,33] demonstrated that a partially reduced form of the enzyme was air-stable . Forward titration with NADPH and back titration with $K_3Fe(CN)_6$ led these investigators to propose that the air-stable form of the enzyme contained two electrons

per mole of enzyme (two flavins) and that the catalytic cycle of the enzyme involved the oscillation of the enzyme from the partially reduced to fully reduced state. Iyanagi and Mason [34] showed that the air-stable, partially reduced form of the reductase contained only one electron per mole of enzyme. They showed with epr (electron paramagnetic resonance) that the air-stable, partially reduced form of the enzyme contained 38-51% of the flavin in the detectable semiquinoid state . The conclusion was justified that this form contained one flavin in the fully oxidized state and the other half reduced. Other investigators soon followed in confirming this conclusion also for the detergent solubilized reductase [36,49]. Additional studies have further elucidated the oxidation/reduction characteristics of the reductase. The potentiometric and spectrophotometric titration curves obtained by reducing the trypsine-solubilized reductase with dithionite under anaerobic conditions [50] have been analyzed by computer generated titration curves for possible redox mechanisms. Iyanagi [50] postulated from these measurements that the reduction occured in four single electron reduction steps whose midpoint potentials are :-0.110,-0.270, -0.290 and -0.366 V. Refinement of the mechanism, including details of reduction by NADPH and reoxidation by cytochrome P-450 , led the investigators to suggest the possibility of intramolecular transfer of reducing equivalents between flavin molecules. This mechanism would allow for the full reduction of one flavin, the transfer of both reducing equivalents to the other flavin and the subsequent transfer from this flavin to various electron acceptors. As before it was soon thereafter also proved to be the same for the detergent solubilized reductase [51]. Fig. 5A and 5B show absorption spectra obtained during reduction of the enzyme. The experiments described by Iyanagi indicate that the two flavins have different redox potentials but did not identify the midpoint- potentials to the individual flavins. The answer to these questions came from Vermillion and Coon [27]. They prepared a FMN-depleted reductase and measured the redox characteristics of this sample. Anaerobically reduced FMN-depleted reductase was shown to be oxidized by $NADP^+$, indicating a midpoint potential for the FAD near the midpoint potential of the NADPH/$NADP^+$ couple. The one electron reduced form of the FMN-depleted reductase (air-unstable) exhibits spectral charateristics of the semiquinone form of the low potential flavin of the native enzyme. These data allow the assignment of

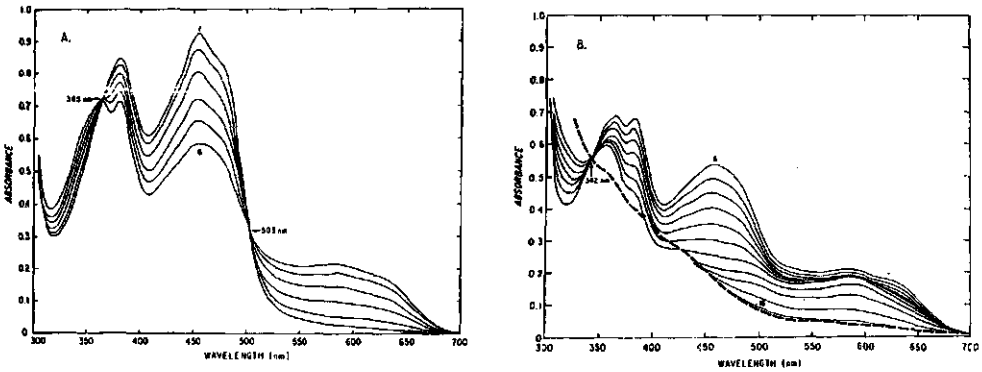


FIGURE 5 A,B
Anaerobic titration of NADPH-cytochrome P-450 reductase with dithionite : spectrophotometric results. NADPH-cytochrome P-450 reductase, 86 μM in 0.1 M potassium phosphate, pH 7.7, was titrated at 25°C in the presence of methylviologen (2 μM) with sodium dithionite (7 mM) dissolved in 0.01 M potassium phosphate, pH 8.35. The experimental curves were not corrected for dilution. Parts A and B represent a single continuous titration but sets of curves are seperated in the two graphs to show two individual sets of isosbestic points occurring during the titration. The dashed line Fig.5B represents fully reduced reductase. Obtained from Iyanagi [50].

FAD as the low potential and FMN as the high potential flavin of the reductase.

Iyanagi proposed in 1974 [50] a scheme (Fig. 6) for the reduction of reductase by NADPH. Experiments executed by Yasukochi [49] and Masters [52,53] have produced evidence for such a mechanism of reduction of the reductase by NADPH. Their stopped flow experiments indicate the initial rapid formation in which one of the two flavins is fully reduced whilst the other is oxidized. This two electron requiring step is reported to have a rate constant of 180 sec^{-1} and is followed by a slower reaction in which the air-stable semiquinone is formed (k= 0.026 sec^{-1}), producing superoxide anion, O_2^- , in the presence of O_2. The evidence from various laboratories is consistent with a differential role for the two flavins in the native enzyme. The FAD site is indicated as the site of interaction with the electron donor based on the reducibility of the FMN-depleted enzyme and the demonstration that electron transfer from FAD to FMN is thermodynamically favourable [25]. The assignment of FMN as the exit site of the electrons is proven by the dependence of the reduction of cytochrome P-450 on the presence of FMN [25,36]. The pathway of electrons through the reductase is now thought to be NADPH --→ FAD --→ FMN --→ acceptors.

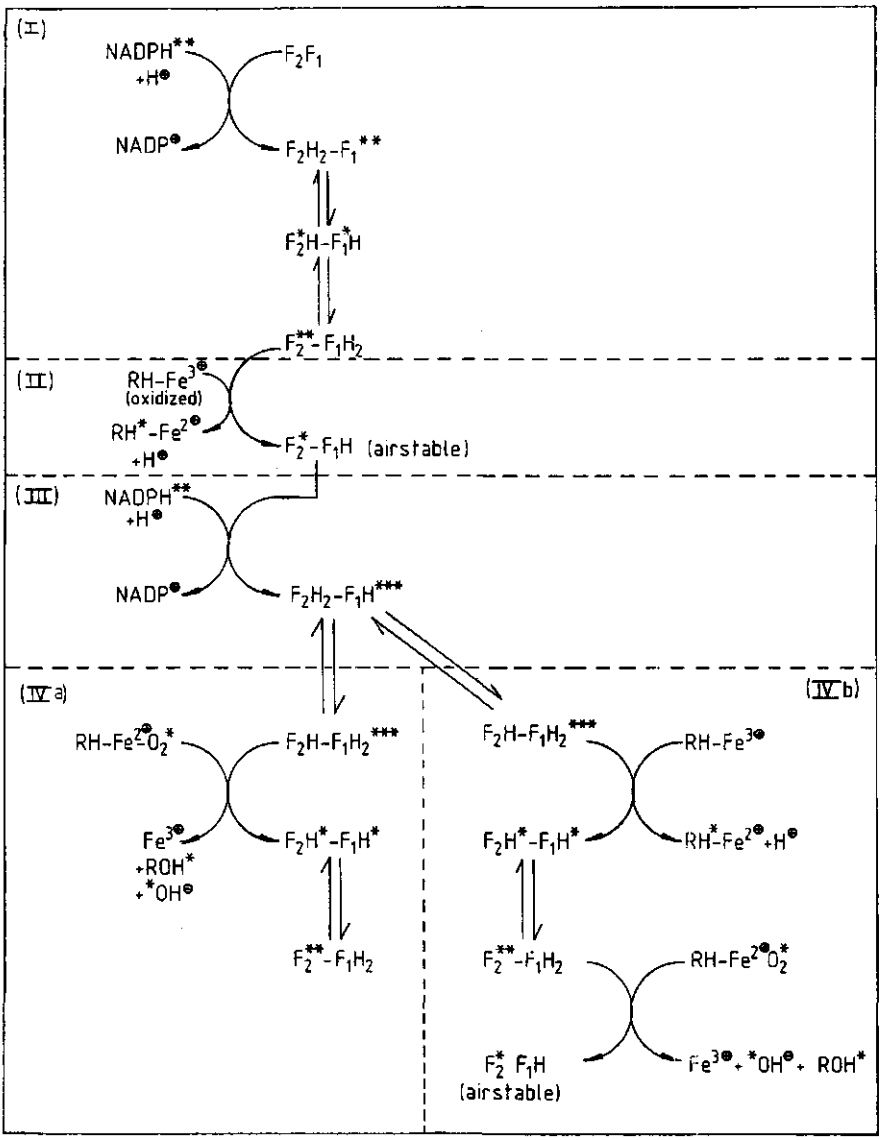


FIGURE 6
Scheme proposed by Iyanagi [50] for the reduction of NADPH-cytochrome P-450 reductase by NADPH.

Physical properties:

Nisimoto et al [54] suggested from photochemically induced dynamic nuclear polarization (CIDNP) studies that a tyrosine residue could be located in the vicinity of the FMN-binding domain which constitutes part of the active center of the reductase. Sugiyama [55] explored by Raman spectroscopy the interactions of the two flavins with their surrounding binding sites in various redox states of the native enzyme and FMN-depleted protein. Their results indicate that, whereas the oxidized forms of the two flavins give identical spectra suggestive of similar binding domains, the spectra of the FADH- vs FMNH- semiquinone forms show distinct shifts that indicate differences in their interaction with their environments.

CD spectra of the reductase were published by French and Coon [56] in 1979 and by Knapp et al. [39] in 1977. Both calculated a 29% alpha helical content of the reductase although on reductase from different species (resp. rabbit and rat liver). EPR spectra were published by Iyanagi and Mason [34]. The g=2.00 signal observed in the semiquinone state confirmed the presence of a flavin radical. P^{31}-NMR measurements [57] indicated that the free radical in the semiquinone state of the enzyme was located on the FMN. Blumberg et al. [58] reported on time-resolved fluorescence studies on the enzyme in which they postulated that energy transfer between the two flavins possibly occurs.

Chemical modification:

To study the essential SH groups in the reductase several experiments were performed. Lazar et al [59] chemically modified the reductase with 5,5'-dithio-bis(2-nitrobenzoate) (DTNB). They showed that one SH group was protected from modification by NADPH binding suggesting its vicinity to the NADPH binding site. Grover and Piette [60] labeled the sulhydryl groups of the reductase with spinlabeled p-hydroxychloromercuri benzoate, the spinlabel was 2,2,6,6-tetramethyl-4-hydroxypeperidine-1-oxyl. They probed the environment of these SH groups as a function of changes in the protein's physical environment known to affect its catalytic activity. Seven SH groups could be spinlabeled. One or two of the sites labeled with PCMB-SL are in close proximity to the protein's NADPH binding site and cause a substantial loss of FAD from the protein. The same conclusions can be drawn from the experiments of Nisimoto and Shibata [61,62]

who modified the seven SH groups of the reductase with PCMB. Vogel et al. [45] used a different SH modifying agent: monobromobi-mane (4-(bromomethyl)- 3,6,7- trimethyl- 1,5- diazabicyclo[3.3.0]octa- 3,6- di-ene-2,8-dione). They sequenced the CNBr fragments of the reductase after labeling in the presence and absence of NADPH. Two cysteine residues are protected from labeling by NADPH. Making use of the sequence described by Porter and Kasper [44] these two cysteines were identified as cys-472 and cys-566.

Another amino acid which has been modified is lysine . Inano and Tamaoki [63] modified the enzyme with TNBS (sodium 2,4,6-tri-nitrobenzene sulfonate). They showed that one lysine residue was protected from modification by NADPH or 2'AMP binding. From their results they proposed that one residue of lysine is located at the binding site of the 2'-phosphate group of the adenosine ribose of NADPH and plays an essential role in the catalytic function of the enzyme.

Molecular biology:

Another approach to study NADPH-cytochrome P-450 reductase is by modern molecular biology techniques, such as gene cloning, gene expression and cDNA synthesis.

The group of Kasper et al. [44,64-66] succeeded to isolate the mRNA on which the reductase was encoded and cDNA was synthesized. After sequencing the DNA gene of the reductase the amino acid was deduced. It agreed very well with other sequences derived from direct amino acid sequence analysis. Comparison with other flavoprotein amino acid sequences [48] showed some homology and was ascribed to the FMN- and FAD binding domains. At the same time Murakami et al. [47] cloned the cDNA for rat liver NADPH-cytochrome P-450 reductase. Katagiri [46] cloned the cDNA for rabbit liver NADPH-cytochrome P-450 reductase. The nucleotide sequence of this cDNA showed 85% homology to that of the rat reductase [44,47].

Three expression plasmids were constructed and introduced into Saccharomyces cerevisiae AH22 cells. These yeast cells were able to produce an active NADPH-cytochrome P-450 reductase system. Recently Murakami [67] published an article on the construction of the functional fused enzyme between rat cytochrome P-450c and NADPH-cytochrome P-450 reductase. Porter et al. [68] reported on the expression of the 78 kDa protein in

E.coli.

The presence of clones of the reductase gene led to unprecedented possibilities. One of them is site-directed mutagenesis with which it is possible to substitute individual amino acids which are of catalytic importance.

Protein/flavin and protein/cytochrome P-450 (c) interaction:

To study the protein-flavin interaction it was necessary to obtain a protein sample which was free of one of the two flavins present in the reductase. Vermillion and Coon [27] were successful in obtaining a FMN-depleted enzyme which could be reconstituted to an active enzyme again. They showed that also some other flavins besides FMN could restore cytochrome c reduction activity of the FMN-depleted reductase.

In 1986 Kurzban and Strobel [69] succeeded to release FAD from the enzyme and to reconstitute it again to an active enzyme. This leads to more opportunities to study the FAD apo-protein interaction.

The interaction of NADPH-cytochrome P-450 reductase with his native electron acceptor cytochrome P-450 has been studied in great detail [70-79]. A rigid arrangement of reductase and cytochrome P-450 ('cluster') in the membrane was postulated [70] whereas others proposed an interaction by random collisions [71,72]. Recent studies using purified enzymes have provided evidence that the reductase and cytochrome P-450 LM2 form a catalytically significant complex when the two proteins are mixed in a 1:1 stoichiometric ratio [73,76-79].

Nisimoto localized the cytochrome c binding domain on the enzyme by cross linking studies with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide at low ionic strength [80]. He proposed that Asp-207,Asp-208,Asp-209,Glu-213,Glu-214 and Asp-215 are in exposed location near the area where one heme edge comes close to the molecular surface.
It has been proven that phospholipids are necessary to reconstitute the reductase and the cytochrome P-450 to an active system [32,73-75,81,82].

Presence and localization:

The presence of the reductase in different organs and species has been demonstrated. The enzyme has been purified from or has been proved to be located in : rat liver [21,22,25,32], rabbit liver [34,56], pig liver

[41,42,45], rabbit lung [83], human placenta [84], Jeruzalem-artichoke (Helianthus tuberosus L.) tubers [85], swine testis [86], rat nasal mucosa [87], rat and monkey central catecholaminergic neurones [88] and Acinetobacter calcoaceticus [89].

Aim of this study:

NADPH-cytochrome P-450 reductase is an very interesting flavoprotein, because it contains two different flavines, FAD and FMN. Because very little knowledge exists on the biophysical properties of the enzyme, we started this study in order to get a better understanding on how electrons are transported from NADPH to FAD, from FAD to FMN and from FMN to cytochrome P-450. Are the flavins in close contact with each other for electron transport? Are the flavins in the enzyme unique or can they be replaced by other? To answer these structure-function questions some biochemical and biophysical studies were done on the native reductase, on the FMN-depleted reductase and on the FMN-reconstituted FMN-depleted reductase.

Chapter 1 gives a extensive review on the NADPH-cytochrome P-450 reductase. History, purification, presence, localization, oxidation /reduction properties, amino acid and sequence analysis, physical properties, chemical modification, flavin-protein interaction, interaction reductase-cytochrome P-450 (c) and molecular cloning are some topics that are discussed.

Chapter 2 deals with a P^{31} NMR study on the enzyme. Every phosphorus atom in nature is P^{31} and can be observed by the NMR (nuclear magnetic resonance) technique. The reductase contains two flavins, FAD and FMN (Fig. 1), which possess respectively two and one phosphate group. The environment of these phosphates can be monitored with P^{31} NMR in the different redox states of the enzyme. Other phosphorus atoms present in the sample can be observed and their origin can be deduced. Information can be obtained if they are located on the surface of the protein by accessibility for the solvent.

Chapter 3 shows that time resolved fluorescence technique is a powerfull tool to study the flavin protein interaction. The great sensitivity of the equipment used led us to observe the fluorescence of the flavins which are quenched to a great extent. With very short excitation pulses the fluorescence deay can be observed on a subnanosecond time scale. This

means that small differences in the flavin surrounding can be observed. The fluorescence and anisotropy decay parameters obtained after a fit procedure of the experimental decay curves led us to postulate a model of energy transfer between the two flavins and the spatial relationship between the FAD and the FMN.

In Chapter 4 we studied the rate of recombination of different (modified) flavins. As was shown before, FMN can be removed from the protein and can be reconstituted to an active enzyme again. We checked some other flavins for their ability to restore activity and how fast the proces of reassociation occurs.

Chapter 5 summarizes a few methods which could be used to make the reductase FMN-depleted in large amounts. Results of these procedures are presented. With several biophysical techniques the holo-, FMN-depleted and FMN-reconstituted enzyme has been studied : P^{31} NMR (nuclear magnetic resonance), CD (circular dichroism), UC (ultracentrifugation), steady state fluorescence and time resolved fluorescence.

A summary chapter completes this thesis.

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CHAPTER 2

NADPH-CYTOCHROME P-450 REDUCTASE : A PHOSPHORUS-31 NMR STUDY

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SUMMARY

The microsomal enzyme, NADPH-cytochrome P-450 reductase has been studied by ^{31}P NMR techniques. It is shown that in the native enzyme (78 kDa), besides the phosphate resonances of the two prosthetic groups, FAD and FMN, also additional resonances are observed. The resonances are shown to belong to tightly but noncovalently bound 2'AMP and phospholipids. The amount of phospholipids present varied depending on the preparation used, but the 2'AMP phosphorus resonance was observed in every preparation.

Addition of Mn^{2+} to the oxidized enzyme caused the resonances of the bound phospholipid molecules to broaden beyond detection and also led to a 70 Hz broadening of the resonance of the phosphate group of 2'AMP . These results suggest that the phosphate groups of the phospholipid molecules and of the 2'AMP group are exposed to bulk solvent.

On reduction of the enzyme to the paramagnetic semiquinone state the 2'AMP and FMN resonances are broadened albeit the latter only in the presence of phospholipids, this in contrast to results recently published by Otvos, Krum and Masters (Biochemistry 25, 7220-7228, 1986). It should be noted that Otvos et al used the proteolytic fragment (69 kDa) of the native protein (78 kDa).

Experiments were performed on enzyme preparations isolated from two different sources,i.e. pig liver and rabbit liver. The spectra obtained from both sources agreed very well with each other. The ^{31}P NMR spectrum of FMN-depleted protein changes dramatically as compared to that of native enzyme. The 2'AMP resonance disappears and the resonances of the

pyrophosphate group of the bound FAD are comparable to free FAD. On readdition of FMN the FAD resonances shift towards the position as observed in the native enzyme and the FMN resonance appears at 4 ppm.

INTRODUCTION

NADPH-cytochrome P-450 reductase, in the following abbreviated as reductase for convenience, is a flavoprotein component of the endoplasmatic reticulum of liver and some other organs [1-5]. It catalyzes the transfer of electrons from NADPH to cytochrome P-450 [6-9]. Cytochrome P-450 is the terminal enzyme in the microsomal monooxygenase system which hydroxylates a variety of compounds such as alkanes, fatty acids, drugs, steroids and xenobiotics [10-12]. The reductase contains 1 mole of FAD and FMN per polypeptide chain of molecular mass of 78.000 Da [6-9,13]. By proteolytic treatment a 69 kDa enzyme can also be obtained [14]. The two flavins play an important role in the electron transfer process to cytochrome P-450. The electrons enter on the FAD-site, from which they are transported to FMN. FMN transfers the electrons to cytochrome P-450. During this electron transfer FAD and FMN occur in different redox states.

^{31}P NMR is a powerful technique to study phosphorus atoms in proteins. Matheis and Whitaker [15] reviewed the ^{31}P NMR chemical shifts of covalently bound phosphates to proteins. Many proteins possess covalently bound phosphates and their presence is not always understood. The NMR technique has also been used to study the flavin coenzyme and protein-bound phosphorus residues: flavodoxin from Megasphaera elsdenii [16], glucose oxidase from Aspergillus niger [17], xanthine oxidase from buttermilk [18] and flavodoxin from Azotobacter vinelandii [19] are some examples of flavin enzymes which were studied by ^{31}P NMR.

Otvos et al [20] investigated the reductase by ^{31}P NMR . We started independently the same type of experiments before their paper was published. However, some important differences exist between their results and ours. The most prominent point of difference is the observation of Otvos et al. [20] that the phosphorus resonance of FMN in the semiquinone state of the protein is not or only very little broadened, but decreased slightly in intensity in contradiction to our preliminary published results [21]. Since Otvos et al. [20] used the protease-treated reductase the different observations in the two groups could be due to

the particular preparation used. In addition, as far as FMN-dependent flavoproteins are concerned which have been studied by ^{31}P NMR techniques, the observation that the resonance of the phosphorus atom of FMN is not be influenced by the semiquinone would be an unprecedented case in this special field. In order to clarify this point and to contribute to a better understanding of the structure-function relationship of NADPH-cytochrome P-450 reductase we continued our study. In this study we compared the ^{31}P NMR properties of various preparations of the enzyme from different sources. It was found that native enzyme (78 kDa), containing strongly bound phospholipids, shows the expected broadening of the resonance of FMN. This broadening is however diminished when the phospholipids are removed from the protein. This process is reversible and is dependent on the preparation of the enzyme, i.e. 78 and 69 kDa. This observation is discussed briefly with respect to the possible function of phospholipids in the enzymic properties of NADPH-cytochrome P-450 reductase. Preliminary results were published elsewhere [21,22].

MATERIALS AND METHODS

NADPH-cytochrome P-450 reductase was purified from liver microsomes of phenobarbital-treated rabbits and from liver microsomes of untreated pigs by detergent solubilization, DEAE-cellulose ion exchange chromatography and affinity chromatography on 2'5'-ADP Sepharose 4B by procedures described before [8], except for some small modifications. The resulting preparations were homogeneous as judged by sodium dodecyl sulphate polyacrylamide gel electrophoresis which was carried out according to Laemmli [23]. Some preparations showed besides the holoprotein of 78 kDa some minor protein impurities on SDS gel electrophoresis. These are due to proteolysis of the native enzyme. Protein concentrations were estimated by the method of Lowry et al [24]. Reductase activity was measured at 25°C in 300 mM potassium phosphate buffer, pH 7.7. Rates of reduction of cytochrome c by NADPH were followed by monitoring the absorbance changes at 550 nm with $\Delta\epsilon$ = 21.000 $M^{-1}cm^{-1}$.

Reductase concentrations were determined spectrophotometrically using a molar extinction coefficient of 21.4 $mM^{-1}cm^{-1}$ at 450 nm. Enzyme samples were taken up in 50 mM Tris/HCl, containing 20% glycerol, 0.1 mM DTT, pH 7.7. To remove inorganic phosphate, samples were brought on a Biogel

P-6DG column. The samples were concentrated to the desired concentration by ultrafiltration on an Amicon apparatus (YM-30 filter).

FMN-depleted reductase was obtained by repetitive ultrafiltration/concentration on an Amicon apparatus (YM-30 filter) using 100 mM Tris/HCl, pH 8.4, containing 20% glycerol, 1.0 mM DTT, 0.1 mM EDTA and 2 M KBr.

Reconstitution was done with an excess of FMN in 20 mM potassium phosphate , pH 7.7, containing 10% glycerol, 0.1% lubrol, 0.1 mM DTT, 0.1 mM EDTA for several minutes at 25°C. Excess flavin was removed by gel filtration.

The 78 kDa reductase was split into the 69.000 Da fragment by adding trypsine and incubation for 1 hour at 4°C. No cytochrome c reduction activity was lost during incubation.

The reductase was reduced to the semiquinone state by adding 1 equivalent of sodium dithionite in the presence of air. The four electron reduced state was achieved by adding 4 equivalents of sodium dithionite anaerobically. The reductase was reoxidized by adding $K_3Fe(CN)_6$ prior to applying the enzyme on a Biogel P-6DG column to remove the metal ions.

Phosphorus concentrations were determined by the procedure outlined by Bartlett [25]. The FAD and FMN contents were determined as described by Wassink and Mayhew [26] using phosphodiesterase to convert FAD into FMN.

The NMR samples contained 10% D_2O for locking the magnetic field. ^{31}P-NMR spectra were recorded at 120.8 MHz on a Bruker CXP300 spectrometer , equipped with an Aspect 2000 computer. Quadrature phase cycling was employed. All spectra were obtained using 10 mm precision NMR tubes (Wilmad). The chemical shifts were determined relative to the external standard of 85% phosphoric acid. Spectra were obtained at 17±2°C and applying a 0.5 W broad-band proton decoupling. The following instrumental settings were used in the acquisition of the free induction decays : 10 μs pulse width (30° pulse), 30 μs delay time, 4800 Hz spectral width, 1.1 s acquisition time, 8 K data points.

RESULTS

NADPH-cytochrome P-450 reductase contains 1 mole of FAD and 1 mole of FMN per mole of enzyme [6-8,27-31]. In all our preparations we ascertained by analysis that the FAD/FMN ratio was about 1:1.

Fig.1A shows the ^{31}P NMR spectrum of the oxidized form of an preparation of pig liver reductase containing about equal amounts of 78 kDa and 56 kDa reductase. The 56 kDa part of the enzyme is a proteolytic cleavage product already mentioned by Haniu et al.[32,33]. The spectrum shows more resonances than expected on the basis of the flavin content of the enzyme. The two high field resonances at -7.4 and -11.3 ppm are assigned to the pyrophosphate moiety of FAD. The peaks are rather broad ($\Delta\nu_{1/2}$ =100 Hz) which suggests that the FAD prosthetic group is tightly bound. The two resonances around 0 ppm (-0.3 and 0.4 ppm) arise probably from phospholipids bound to the protein. This suggestion is supported by the fact that addition of about 1/2 equivalent of phosphatidylcholine (with respect to the protein concentration) to the sample caused a strong increase of the resonance at -0.3 ppm. Binding of phospholipids by the

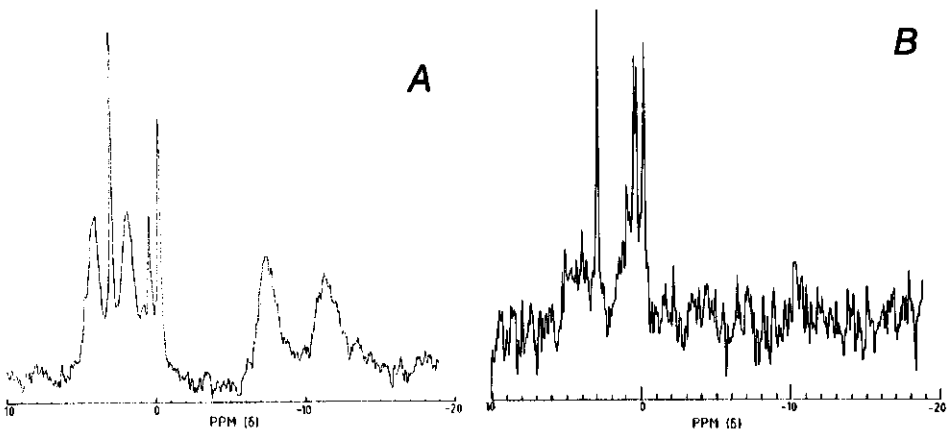

FIGURE 1: 120.8 MHz ^{31}P NMR spectra of NADPH-cytochrome P-450 reductase from pig liver. The enzyme concentration was 365 µM, based on flavin content. The enzyme was dissolved in 50 mM Tris/HCl, pH 7.7, containing 20% glycerol, 0.1 mM DTT. The temperature was 17°C. A) Oxidized native enzyme (78 kDa,56 kDa), 206.278 acquisitions.
B) as in A, denatured enzyme, 79.691 acquisitions.

reductase has also been demonstrated by others [34,35]. During our work we consistently observed that enzyme preparations in the native state

(78 kDa) always contained a variable content of phospholipid as demonstrated by ^{31}P NMR and chemical analysis. It is very difficult to release phospholipids from the protein, but they can partly be removed by extensive washing of a protein preparation bound to a 2'5'ADP-Sepharose column. Therefore it must now be concluded that phospholipids interact very strongly with the reductase, but the phospholipids are non-covalently bound (see below). In this context it is interesting to note that an incidental denaturation of the preparation at a later stage of this study showed that the irreversibly denatured protein still contained the phospholipids (Fig.1B). This spectrum also shows that the phospholipid resonance is sharper and better resolved than in the spectrum of the native protein. These results also demonstrate that probably different phospholipid molecules are bound to the reductase or one particular phospholipid is bound to different sites of the protein, i.e. in different microenvironments.

The relatively sharp peak at 2.9 ppm (Fig. 1A) is assigned to free phosphate, which was a contamination of the buffer used. ^{31}P NMR spectra completely free of free phosphate were also obtained (see below).

The peaks appearing at 1.8 ppm and 4.1 ppm in the ^{31}P NMR spectrum (Fig. 1A) are assigned to 2'AMP and FMN, respectively, in accordance with the assignments made already by Otvos et al. [20]. However, we have corroborated these assignments by additional experiments, as will be shown below.

The spectrum shown in Fig. 1A exhibits an excellent signal-to-noise ratio considering the size of the protein. Therefore the small peaks seen at about 0.8 and 2 ppm must be considered real, but have not been assigned. Furthermore the resonance of FMN exhibits a shoulder at the low field side of the resonance line. This shoulder could represent the presence of a small amount of free FMN in the preparation. The same observation was made by Otvos et al. [20]. This indicates, depending on the concentration of the reductase used, that all preparations of reductase always contain some free FMN in accordance with its relatively easy removal from the protein.

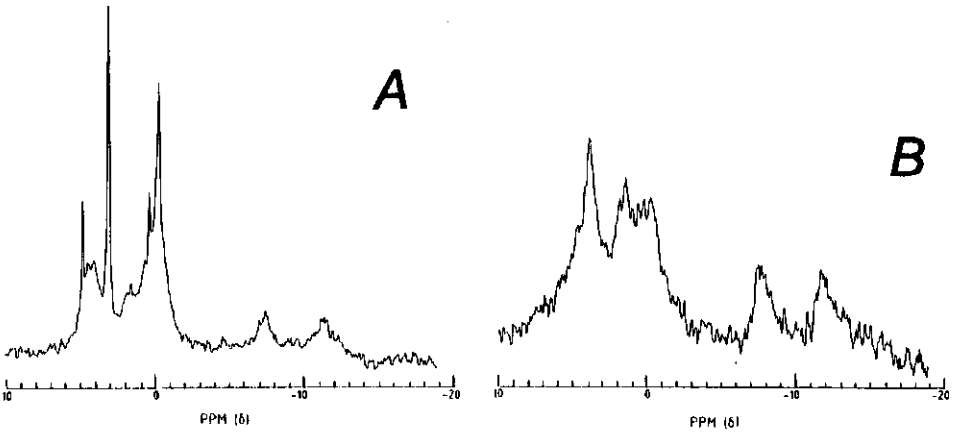

FIGURE 2: 120.8 MHz ^{31}P NMR spectra of NADPH-cytochrome P-450 reductase from rabbit liver. The enzyme concentration was 390 μM, based on flavin content. The enzyme was dissolved in 50 mM Tris/HCl, pH 7.7, containing 20% glycerol, 0.1 mM DTT. The temperature was 17°C. A) Oxidized native enzyme (78 kDa, 69 kDa), 82.632 acquisitions.
B) as in A, + 10% Mn2+ 82.632 acquisitions.

To probe the solvent accessibility of the different phosphate groups in biological material, commonly Mn^{2+} is used as a paramagnetic ion. Adding one tenth equivalent of Mn^{2+} with respect to the reductase concentration to the protein, the presence of Mn^{2+} in reductase affects the resonance lines of phospholipid and free phosphate, which are almost completely broadened and hardly visible anymore in the ^{31}P NMR spectrum (Fig. 2A,2B). In addition the linewidth of the resonance line due to 2'AMP is increased from 80 Hz, in the absence of Mn^{2+}, to 150 Hz in the presence of Mn^{2+} (spectrum not shown). This broadening indicates that the phosphate group of 2'AMP is somewhat accessible to bulk water. The resonance lines of the phosphate groups of FMN and FAD are not at all affected by Mn^{2+} indicating that these groups are burried in the interior of the protein or well protected from access by solvent.

NADPH-cytochrome P-450 reductase forms an "air-stable" semiquinone, which can be obtained by an one-electron reduction of the protein under aerobic conditions. The semiquinone form is exceptionally stable towards oxidation by O_2 and is therefore called "air-stable". From UV spectrophotometric studies it has been suggested that the unpaired electron resides on FMN in reductase [13]. The only technique to prove this assignment is by ^{31}P NMR. Such a spectrum is shown in Fig. 3. The enzyme preparation used in this experiment was the same as used in Fig.1. The semiquinone

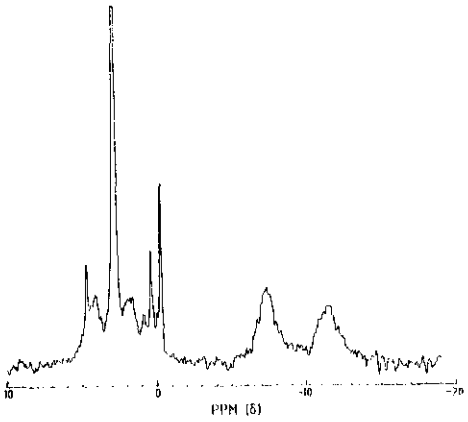

FIGURE 3: 120.8 MHz ^{31}P NMR spectra of NADPH-cytochrome P-450 reductase from pig liver. The enzyme concentration was 365 μM, based on flavin content. The enzyme was dissolved in 50 mM Tris/HCl, pH 7.7, containing 20% glycerol, 0.1 mM DTT. The temperature was 17°C. Semiquinone form (93%) of the sample of Fig.1. 143.961 acquisitions.

content of the sample was 93% of the expected amount. The spectrum (Fig. 3) shows that the FAD resonances remain unaffected by the semiquinone. However, the resonance lines due to the phosphate groups of FMN and 2'AMP are broadened considerably and the intensity of both lines is strongly decreased. This is in contradiction with the results published by Otvos et al. [20]. The shoulder at the low field side of the FMN resonance line is also unaffected by the presence of the semiquinone. As mentioned above this line represents free FMN and is now more clearly observed than in Fig.1A.

The ^{31}P NMR spectrum of the fully reduced (four electrons) reductase is identical with that of the oxidized protein (results not shown, cf. Fig.1A). This indicates that no gross conformational change occurs upon four electron reduction.

The preparation used in Fig.1 and Fig. 3 was composed of equal amounts of 78 kDa and 56 kDa protein. Otvos et al. [20] suggested that the observation of some additional resonances assigned to protein-bound FMN are due to the presence of the 56 kDa fragment in the preparation. Our results clearly show that the inhomogeneity of the sample is not reflected in the ^{31}P NMR spectra. This suggests that the ^{31}P chemical shifts due to FMN in native protein and its 56 kDa fragment are very similar. Both enzymes contain the hydrophobic 10 kDa fragment.

Moreover both proteins apparently form the "air-stable" semiquinone and the semiquinone affects the FMN resonances in both proteins similarly. In order to check if this is really true we also measured pure 78 kDa reductase. This ^{31}P NMR spectrum was identical with that shown in Fig.1A.

The ^{31}P NMR spectra of NADPH-cytochrome P-450 reductase from pig and rabbit livers are very similar. This is an expected result regarding the great structural conservation of the reductase in different species [36]. However the reductase from the different species showed a large variation with respect to phospholipid content and the ease by which they could be removed from the protein.

A ^{31}P NMR spectrum of NADPH-cytochrome P-450 reductase from rabbit liver in the oxidized form (homogeneous preparation, 78 kDa) is shown in Fig. 4A. In contrast to Fig. 1A, this preparation contains a large amount of phospholipid. 2'AMP is present in a lesser amount than in Fig. 1A, also free FMN is clearly seen. Otherwise the spectrum is similar with that of Fig. 1A with respect to the linewidth and position of the different resonances. From this preparation the FMN-depleted reductase was made. The ^{31}P NMR spectrum is shown in Fig. 4B. The spectrum exhibits the resonances due to phospholipid in a resolved manner and the phosphorus resonances of the pyrophosphate group of FAD. The protein-bound FAD shows now, in comparison with that in Fig. 4A, a doublet whose linewidth is much smaller. This result suggests that reductase undergoes a considerable structural change upon removal of FMN. Alternatively the binding interaction between FAD and the protein could also be affected by the removal of FMN from the protein. Reconstitution of FMN-depleted reductase by an excess FMN (24 hours incubation at 4°C) and removal of excess FMN by gelfiltration yielded a preparation whose ^{31}P NMR spectrum is shown in Fig. 4C. Although reconstituted enzyme is enzymatically as active as native enzyme (cytochrome c assay), the ^{31}P NMR spectrum is not identical with that of the native protein. The ^{31}P chemical shifts of the native and the FMN reconstituted FMN-depleted enzyme are identical, the linewidths of the FAD resonance lines are smaller than the corresponding shifts in the native protein, suggesting a less strongly bound FAD. > 5 days after the reconstitution of the FMN-depleted reductase the FAD phosphate resonances are the same as in the native enzyme, indicating that the FAD ultimately returns to its native position (spectrum not shown). The phospholipid resonances in Fig. 4C are much broader than in

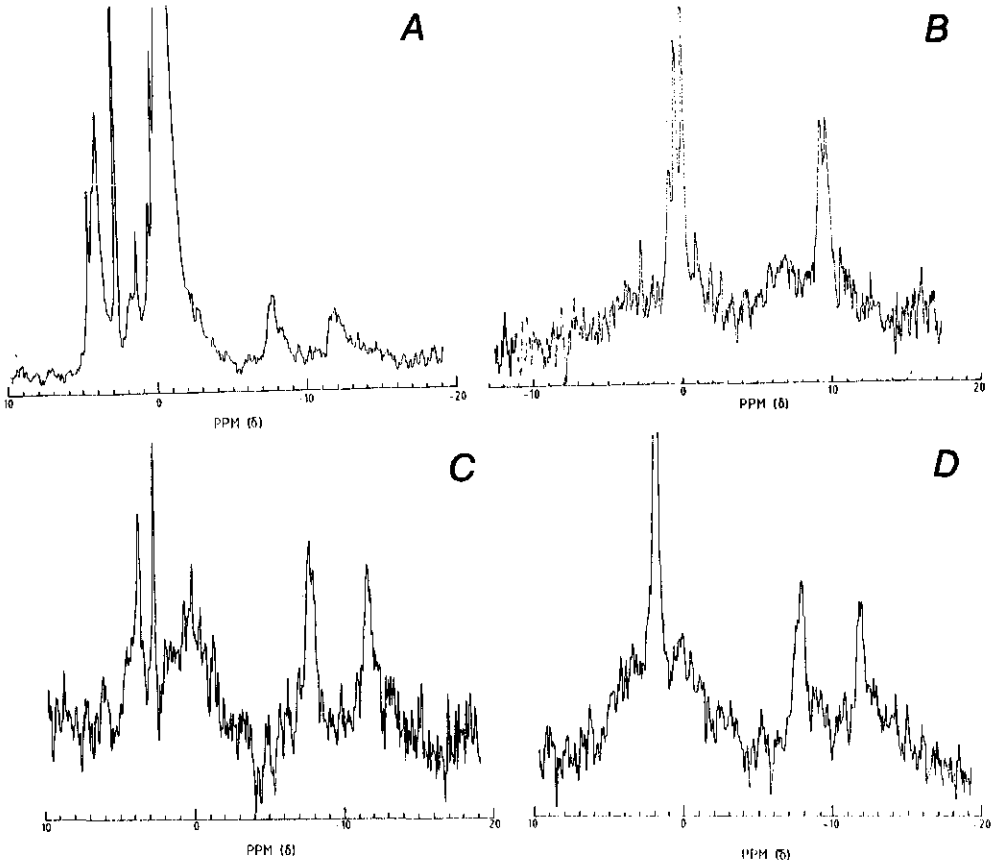

FIGURE 4: 120.8 MHz ^{31}P NMR spectra of NADPH-cytochrome P-450 reductase from rabbit liver. The enzyme was dissolved in 50 mM Tris/HCl, pH 7.7, containing 20% glycerol; 0.1 mM DTT. The temperature was 17°C. A) Oxidized native enzyme (78 kDa). The enzyme concentration was 390 µM, based on flavin content. 109.777 acquisitions.
B) FMN-depleted enzyme of Fig. 4A. The enzyme concentration was 380 µM, based on flavin content. 50.319 acquisitions.
C) FMN-reconstituted enzyme of Fig.4B in the oxidized form. The enzyme concentration was 400 µM, based on flavin content. 39.291 acquisitions.
D) FMN-reconstituted enzyme of Fig.4B in the semiquinone form. The enzyme concentration was 400 µM, based on flavin content. 75.875 acquisitions.

the native protein. The ^{31}P NMR spectrum of the reconstituted reductase in the semiquinone state is shown in Fig. 4D. Again the resonance lines of FAD remain unaffected by the presence of the paramagnetic flavin, but the resonance line due to FMN is strongly broadened, more broadened than that in Fig. 3. Also the phospholipid resonances seem to

be broadened. For the time being we suggest that the larger amount of phospholipid present in the sample shown in Fig. 4A, as compared to Fig. 1A, is responsible for the larger broadening of the resonances due to FMN in Fig. 4D.

The above presented results are not in agreement with those published by Otvos et al. [20]. The only difference between the two sets of data is that we used the native enzyme (78 kDa) whereas Otvos et al. [20] used the proteolytically treated enzyme (69 kDa). In order to study if this difference could explain the two sets of data we prepared the 69 kDa enzyme from the native protein isolated from pig liver. The ^{31}P NMR spectrum of the proteolytically treated enzyme is shown in Fig. 5A. It is apparent that the linewidth of the FAD resonances is decreased as compared to those of the native protein (cf. Fig. 1A), but the resonance position of all lines are not affected. It should also be noted that 2'AMP was added to the enzyme and that the sample is completely free from phospholipid. This is precisely the situation encountered by Otvos et al. [20]. Fig. 5A is very similar with the spectra of Otvos et al. [20]. The ^{31}P NMR spectrum of the protease-treated enzyme in the semiquinone form is shown in Fig. 5B, unexpectedly, but in agreement with the findings of Otvos et al. [20], the resonances of the phosphate group of FMN and 2'AMP

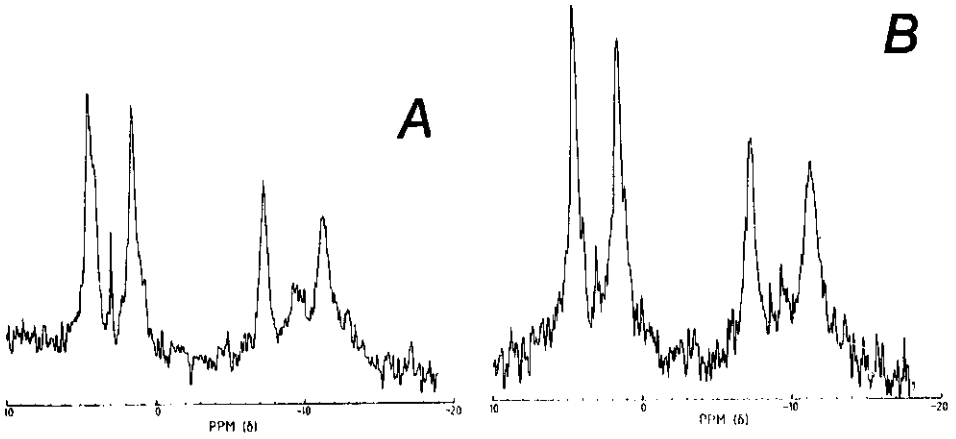

FIGURE 5: 120.8 MHz ^{31}P NMR spectra of NADPH-cytochrome P-450 reductase from pig liver. The enzyme concentration was 345 μM, based on flavin content. The enzyme was dissolved in 50 mM Tris/HCl, pH 7.7, containing 20% glycerol, 0.1 mM DTT. The temperature was 17°C. A) Oxidized enzyme (69 kDa), 61.147 acquisitions.
B) Semiquinone form (78%) of the sample of Fig.4A. 99.999 acquisitions.

are not broadened by the flavosemiquinone, which was present in 78% of the theoretical amount. The results suggest that either the absence of phospholipid or a structural change in the reductase causes the observed difference between the two enzyme preparations.

In order to dicriminate between those two possibilities we tried to make a preparation of 78 kDa reductase which was free of phospholipids. However, we partly succeeded in obtaining such a sample because it turned out to be extremely difficult to remove the phospholipids from the native enzyme without loosing the hydrophobic part. This is in agreement with observations published before [34,35] suggesting a strong interaction between the phospholipids and the hydrophobic part of the reductase. Therefore more or less phospholipid-free preparations of the reductase (78 kDa and 69 kDa) were investigated in the presence and absence of phospholipid. The line widths of the FMN and the 2'AMP resonances are summarized in Table 1. From these results it is apparent that phospholipids are indeed involved. We can consider two possibilities : a) phospholipids cause the association of reductase molecules; b) phospholipids induce a conformational change in the reductase so that the binding sites of the prosthetic groups are altered, i.e. the native state of the protein is regained. More likely is a combined effect of both. We have investigated reductase in the presence of phospholipids by analytical ultracentrifugation studies. It is found that a sample containing only 68 kDa reductase showed the same sedimentationbehaviour in the presence or absence of extra added phospholipids. A preparation of 78 and 69 kDa in a 1:1 ratio showed a different behaviour between the presence and the absence of extra added phospholipids. Therefore we can conclude that phospholipids act on the 78 kDa reductase and influences its sedimentationbehaviour suggesting a conformational change. Association of reductase molecules was not at all observed.

TABLE 1
Linewidths of ^{31}P NMR resonances of NADPH-cytochrome P-450 reductase isolated from pig liver at half maximum heigth

Sample	FMN (Hz)	2'AMP (Hz)
Reductase pig OX few PC	84	47
" " SQ few PC	130	104
" " SQ + much PC	166	138

DISCUSSION

The ^{31}P NMR spectrum of the flavoprotein NADPH-cytochrome P-450 reductase , isolated from pig liver, shows mainly four resonances: -7.4 ppm, -11.4 ppm, 1.5 ppm and 4.0 ppm. The -7.4 and -11.4 ppm resonances are the FAD phosphate resonances. The 4.0 ppm resonance is due to FMN because this peak disappears in the FMN-depleted reductase and reappears in the FMN-reconstituted enzyme and the 1.5 ppm can be assigned to 2'AMP because it reappears after addition of 2'AMP to the FMN-reconstituted reductase. Otvos et al. [20] came to the same conclusions in their experiments, although there are some differences between their and our results. In contrast to Otvos et al. [20] the 2'AMP resonance is more pronounced in all our preparations. The intensity of the 2'AMP resonance suggests equimolar quantity with FMN and FAD. It can clearly be observed (Fig. 1A,3) that on formation of the semiquinone form the 2'AMP and FMN resonances are broadened . Otvos et al. [20] did not observe any broadening of those resonances but observed only a small decrease of the intensity of the FMN resonance.

The difference between the experiments as performed by Otvos et al. [20] and ours are :i) Otvos et al. [20] used the protease cleaved reductase and we the native (78 kDa) reductase; ii) their sample did not contain any phospholipid in contrast to our sample. In view of the fact that we repeated their experiments with the 69 kDa reductase and did not see any broadening or decrease in intensity of the 2'AMP or the FMN resonance on semiquinone formation, the conclusion therefore must be that phospholipid and/or the extra hydrophobic 10 kDa fragment of the reductase is responsible for the observed broadening in Fig. 3. The results in Table 1 support this conclusion.

The broadening observed is a consequence of the increased spin-spin relaxation rate of the phosphorus nuclei under the influence of the unpaired electron i.e. the flavin radical in our case. This relationship is generally described by the Solomon-Bloembergen equation [37,38]:

$$1/T_2 = \frac{1}{15} \frac{\gamma_I^2 g^2 s(s+1) \beta^2}{r^6} \left(4 \tau_c + \frac{3 \tau_c}{1+\omega_I^2 \tau_c^2} + \frac{13 \tau_c}{1 + \omega_s^2 \tau_c^2}\right) \qquad (1)$$

The term containing the modulation of the scalar interaction has been omitted here due to the fact that there is no spin density of the radical

on the phosphorus nuclei. γ_I is the gyromagnetic ratio of the nucleus, s is the nuclear spin, β is the Bohr magneton, ω_S and ω_I are the Larmor precession frequencies of the electron and the nucleus, respectively, τ_C is the correlation time and r is the distance between the nucleus and the electron and is in this particular case the distance between the FMN-phosphate nucleus and the center of the pyrazine ring. In this equation it can safely be assumed that $\omega_S >> \omega_I$, and therefore the last term in eq. 1 can be neglected because $\omega_S^2\tau_C^2 >> 1$.
The correlation time τ_C contains several components, i.e. :

$$1/\tau_C = 1/\tau_S + 1/\tau_r + 1/\tau_m \qquad (2)$$

where τ_S is the relaxation time of the electron, τ_r is the rotational correlation time and τ_m is the lifetime of a nucleus in the bound site. Due to the fact that the flavin radical is tightly bound, the $1/\tau_m$ component can be neglected in the calculation of τ_C.

The observed broadening of the FMN phosphate resonance can be explained in three ways :

a) The phosphate group is closer to the radical in the presence of phospholipids (the term r becomes smaller in the equation).
b) The term τ_C becomes larger.
c) A combination of a) and b).

It can be expected that in Table 1 the line broadening of the FMN resonance in a sample without phospholipids amounts around 30 Hz and that in a sample with phospholipids bound this increases to 80 Hz. In the limit that τ_S can be neglected with regard to τ_r (which is 53 ns for the reductase) one calculates a distance r=13 Å for the sample without phospholipids. This is beyond the limit of a stretched FMN molecule and therefore τ_S has to be taken into account. On the other hand, on assuming a distance r=9 Å, which is about equal to the distance of the middle of the pyrazine ring, where most of the spin density resides, and the phosphorus atom, in a stretched FMN molecule, a value of $\tau_S = 7 \cdot 10^{-9}$ s can be calculated. This is close to a value reported by Palmer et al. [39] ($\tau_S = 10^{-8}$ s). On addition of phospholipids the line broadening increases by about 50 Hz. On assuming no change in τ_S, it can be calculated from eq.1 that the distance r decreases to 7.7 Å. However, on the other hand, assuming no change in r, this leads to an increase of τ_S from $7 \cdot 10^{-9}$ s to $24 \cdot 10^{-9}$ s.

Magdalou [40] reported in a CD-(circular dichroism) study that the mean residue ellipticity at 222 nm increased in the presence of phospholipids from which they concluded that the α-helical content increased from 28% to 41%. A conformational change seems therefore likely and this points to possibility a and c above. We can not discriminate between either two possibilities. However, we favour the possibility that not only a conformational change occurs in the presence of phospholipids but also that τ_c increases due to an increase of τ_s. It is possible that the isoalloxazine ring of the FMN molecule (on which the radical resides) becomes more hydrophobic in the presence of phospholipids and that this result in a different electron spin relaxation time. The fact that phospholipids are necessary for an active reductase-cytochrome P-450 complex and that FMN and cytochrome P-450 should be in each other neigh-bourhood to donate electrons from the FMN side to cytochrome P-450 lead to some interesting possibilities for future research.

The spectrum of the 4 electron reduced reductase is the same as the oxidized one, which shows that the environment of the phosphates did not change upon reduction of the enzyme.

To see which of the phosphates is accessible for the solvent a paramagnetic ion (Mn^{2+}) was added to the enzyme solution which should lead to broadening of those resonances which are attributed to solvent accessible phosphates. One of the main four resonances (1.5 ppm, 2'AMP) showed a 70 Hz broadening so this phosphate is fairly accessible for the solvent. The resonances due to the phospholipids disappear completely so they are accessible for the paramagnetic ion Mn++ and are therefore on the outer surface of the enzyme.

The fact that the 2'AMP resonance shows up throughout all preparations and experiments shows that it is tightly attached to the enzyme.

Comparison of the ^{31}P NMR spectra of reductase isolated from rabbit liver and the one isolated from pig liver resemble each other very much as far as the four major resonances are concerned. The FMN- and FAD-phosphate binding domains are therefore the same in both enzymes. The degree of resonances in the 0 ppm region (phospholipids) vary.

Recently [31] the amino acid sequence was solved and there was a great deal of homology between the rabbit- and pig reductase sequence so it is probable that also the flavin binding domains are homologeous.

Phosphorus analysis agrees very well with the NMR spectrum so that 1

mole of NADPH-cytochrome P-450 reductase contains 4 moles of phosphate : one FMN-, two FAD- and one 2'AMP-phosphates.

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CHAPTER 3

TIME-RESOLVED FLUORESCENCE SPECTROSCOPY ON NADPH-CYTOCHROME P-450 REDUCTASE

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SUMMARY

Fluorescence as well as fluorescence anisotropy decay parameters have been obtained from NADPH-cytochrome P-450 reductase by time-resolved fluorescence spectroscopy. The two flavins in the enzyme, FMN and FAD, are slightly fluorescent and exhibit heterogeneous fluorescence lifetimes, as observed with other flavoproteins.

The time-dependent anisotropy is also composed of at least three components and is wavelength-dependent. Excitation at 514 nm instead at 458 nm yields two correlation times, the shortest decay time upon 458 nm excitation is no longer present in the decay. FMN-depleted NADPH-cytochrome P-450 reductase shows also only two correlation times as does the enzyme in the "air-stable" semiquinone state.

Steady state anisotropy measurements of native and FMN-depleted protein showed that the former exhibits lower values in the region 420-480 nm than the latter, but at wavelengths > 500 nm the anisotropy becomes equal in both preparations. A similar situation was encountered in model compounds, monomeric and dimeric flavins, immobilized in polymethyl metacrylate.

From the results various geometric parameters were calculated. These data indicate that energy transfer occurs between the prosthetic groups in NADPH-cytochrome P-450 reductase and that the distance between the two flavins is about 1 nm.

The results are briefly discussed with regard to the biochemical significance of the data.

INTRODUCTION

NADPH-cytochrome P-450 reductase from liver is an unusual mammalian flavoprotein that contains two prosthetic groups, i.e. FMN and FAD [1-3]. The native membrane-bound protein possesses a molecular mass of 78 kDa. By proteolytic treatment of the protein a 68 kDa fragment of the enzyme can be obtained in a pure form. This reductase is no longer able to transfer electrons from NADPH to cytochrome P-450 but it still catalyzes the electron transfer to the artificial acceptor cytochrome c. Obviously the hydrophobic part of native reductase, which is lost upon proteolysis, is responsible for the proper interaction with cytochrome P-450. A FMN-depleted form and a rather stable semiquinone, in the literature referred to as "air-stable" radical, can be formed from both enzyme preparations. This suggests that the basic chemical and structural properties of reductase are not affected when treated with proteases.

Although NADPH-cytochrome P-450 reductase plays an important role in the metabolism of xenobiotics only little structural information is yet available. An outstanding problem in NADPH-cytochrome P-450 reductase is that we still know relatively little about the spatial relationship between the two flavins. There are only a few physical techniques which could shed light on this problem. However only a few physical studies have been carried out with NADPH-cytochrome P-450 reductase, i.e. a resonance Raman [4] and a ^{31}P NMR study [5]. The resonance Raman study [4] revealed that the interactions of the flavin(s) with the protein in the native and FMN-depleted form are identical. These interactions are altered in the semiquinone state of the protein. The ^{31}P NMR study [5] proved unequivocally that the unpaired electron in the "air-stable" semiquinone form of the reductase resides on FMN in agreement with suggestions deduced from light absorption studies [6-8]. A technique more suitable to obtain the desired information is time-resolved fluorescence. Such a study was carried out by Blumberg et al [9]. It was concluded that energy transfer possibly occurs between the two flavins in the reductase. However the experimental data obtained at that time did not allow to draw firm conclusions from the data. The laser fluorescence technique has undergone since a rapid development and reached a high degree of sophistication. Owing to the high dynamic range of intensity and good time resolution of such instrument very weakly fluorescent compounds

can be investigated and the relevant dynamic information can be extracted from the experimental results by computational methods.

Therefore we decided to reinvestigate NADPH-cytochrome P-450 reductase by this technique in order to obtain more detailed information about the existence or absence of energy transfer between the two flavins in the enzyme. The results are also of general interest for the understanding of the mechanism of electron transfer catalyzed by flavoproteins, for example to answer the question : must there be interflavin contacts for electron transfer to occur ?

In this paper it is shown that the protein shows complex time-resolved fluorescence properties. Comparing different enzyme preparations it was concluded that indeed energy transfer between the two flavins takes place. The results are interpreted with the aid of different models. Preliminary results of this study have been published elsewhere [10,11].

MATERIALS AND METHODS

Isolation of the enzyme:

NADPH-cytochrome P-450 reductase was isolated from liver microsomes of phenobarbital-treated rats and purified by a published procedure, except for minor modifications [12]. After solubilization of the microsomes, the enzyme was purified by DEAE ion-exchange chromatography followed by 2'5'-ADP-Sepharose affinity chromatography. As judged by SDS-PAGE according to Laemmli [13] the reductase was homogeneous. The molecular mass of the enzyme was 78 kDa, as reported before [12,14-16]. Activity was measured at 25°C in 0.3 M potassium phosphate, pH 7,7, by monitoring the reduction of cytochrome c at 550 nm ($\Delta\epsilon$ = 21.0 $mM^{-1}cm^{-1}$).

Preparation of FMN-depleted enzyme:

1 ml of a 30 µM stock solution of NADPH-cytochrome P-450 reductase was diluted into 400 ml "apo-buffer" (100 mM Tris/HCl, pH 8.4, containing 20% glycerol, 2 M KBr, 0.1 mM EDTA, 1.0 mM DTT, 0.1 mM PMSF) and concentrated in an Amicon ultrafiltration apparatus (400 ml; YM-30 filter) at 4°C. After concentration of the sample it was again diluted with "apo-buffer" and concentrated. This procedure was repeated several times until FMN was completely removed. After the

final concentration step KBr was removed from the FMN-depleted enzyme by gelfiltration (Biogel P-6DG, 25 mM potassium phosphate, pH 7.7, containing 20 % glycerol, 0.1% lubrol, 0.1 mM DTT, 0.1 mM EDTA). The content of residual FMN in the sample was analyzed according to the procedure described by Mayhew and Wassink [17]. The activity of the FMN-depleted reductase was measured with and without incubation with FMN.

Instruments:

The time resolving fluorimeter consisting of an Ar-ion laser , associated optics and electronics, was extensively described earlier [18-20]. Excitation wavelengths used were 458 nm or 514 nm; the emission wavelengths were 531 or 550 nm. Measurements were carried out at 10°C.

Steady state fluorescence:

Steady state fluorescence was measured by a instrument described by Jameson et al. [21]. Excitation band width was 2 nm. Emission wavelengths were selected by Corning 3-67,3-69 and 4-94 filters. Measurements were performed at 10°C and 15 measurements were averaged. Fluorescence quantum yields were calculated with FMN as reference (quantum yield FMN = 0.30 [22]).

Samples and sample preparations:

The samples were measured in 25 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 0.1 mM DTT.

For recombination of the FMN-depleted enzyme with FMN, the protein was incubated with an excess of FMN at 4°C for 1 h. Excess of FMN was then removed by gelfiltration. The semiquinone (one-electron reduced state) of the native reductase was made by addition of small amounts of a dithionite solution under anaerobic conditions. The reduction was followed spectrophotometrically to ensure complete formation of the semiquinone. The solution was made anaerobic by flushing with N_2 or Ar for about 15 min.

The model compounds 3-methyllumiflavin , N(10),N(10)'-propylene- and N(10),N(10)'-hexylene-bis-7,8-dimethylisoalloxazine were prepared according to published procedures [23,24] . The incorporation of these compounds into polymethyl metacrylate has been described previously [25].

As a reference compound erythrosine B (Eastman-Kodak) in water was used. Methanol was fluorescent grade from Merck.

Viscosity:

The viscosity of the buffer was measured at 10°C with a standard viscosity meter.

Data analysis:

Data analysis for the fluorescence and anisotropy decays was performed with an iterative deconvolution method [26] using erythrosine B in water as reference compound.

The decay of fluorescence I(t) and anisotropy r(t) were fitted with multi-exponential functions :

$$I(t) = \sum_i \alpha_i \, e^{-t/\tau_i} \qquad (1)$$

$$r(t) = \sum_i \beta_i \, e^{-t/\phi_i} \qquad (2)$$

Energy transfer:

Electronic excitation energy can be transferred between appropriately selected chromophores. A general requirement for excitation transfer is that the donor fluorescence spectrum overlaps the acceptor absorption spectrum. For dipole-dipole interaction the specific rate of excitation transfer k_{DA} between an excited donor molecule (D) and an acceptor molecule (A) separated by a distance R (in Å) is given by the Förster equation [27] :

$$k_{DA} = 8.71 \cdot 10^{23} \, J \, \kappa^2 \, Q_f \, \tau_{D_0}^{-1} \, n^{-4} \, R^{-6} \quad sec^{-1} \qquad (3)$$

$$k_{DA} = (1/\tau_{D_0}) \, (R_0/R)^6 \qquad (4)$$

where n is the refractive index of the medium between D and A, Q_f the donor fluorescence quantum yield in the absence of acceptor, τ_{D_0} the fluorescence lifetime of the donor in the absence of acceptor, R_0 the critical distance and J is the spectral overlap integral defined by :

$$J = \frac{\int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda}{\int F_D(\lambda)d\lambda} \qquad (5)$$

where $F_D(\lambda)$ is the fluorescence intensity of the donor at wavelength λ and $\epsilon_A(\lambda)$ is the molar extinction coefficient (in $M^{-1}cm^{-1}$) of the acceptor. κ^2 is an orientation factor which depends on the relative orientations of the donor and acceptor molecules and is defined by:

$$\kappa^2 = [\hat{a}\ \hat{d} - 3(\hat{a}\ \hat{r})(\hat{d}\ \hat{r})]^2 \qquad (6)$$

where $\hat{a}$ is a unit vector along the absorption transition moment of A, $\hat{d}$ a unit vector along the emission transition moment of D and $\hat{r}$ a unit vector along the direction between the centers of A and D. The k_{DA} is determined experimentally by measuring the lifetime of the donor in the absence (τ_{D_0}) and the presence (τ_D) of the acceptor. The relationship can be written as [28] :

$$k_{DA} = 1/\tau_D - 1/\tau_{D_0} \qquad (7)$$

While in principle R could be computed from eq. 3, it has become customary to calculate the so-called Förster critical distance, R_0, the distance where deactivation of the donor by means of energy transfer and deactivation by all other quenching mechanisms are of equal probability :

$$R_0^6 = 8.8 \cdot 10^{-25}\ Q_f\ \kappa^2\ n^{-4}\ J \qquad (8)$$

Interpretation of time-resolved fluorescence anisotropy:

In the enzyme studied there are three possible contributions to the fluorescence depolarization : i) the overall motion of the protein, ii) the motional freedom of the flavins and iii) energy transfer between the two flavins.

If two or more independent processes contribute to the total time-dependent fluorescence depolarization, the fluorescence anisotropy can be described by the so-called Soleillet product [29]:

$$r(t) = r(0) \prod_j (3/2 \langle \cos^2 H_j(t) \rangle - 1/2) \qquad (9)$$

where $\langle\ \rangle$ denotes the mean ensemble; $H_j(t)$ the time dependent process described by the angle H_j responsible for depolarization and

r(0) the fundamental anisotropy.

In our case (three contributions at 458 nm excitation) the Soleillet product becomes :

$$r(t) = r(0)\ [(A_w \exp(-t/C_w) + A_{winf})\ (A_t \exp(-t/C_t) + A_{tinf})\ \exp(-t/C_p)] \qquad (10)$$

where $A_w = 1 - A_{winf}$; $A_{winf} = 1/2 \cos H_w\ (1 + \cos^2 H_w)$;
H_w is the top angle of cone diffusion [30]; C_w is the effective correlation time of the "wobbling in cone" motion [30]; C_p is the rotational correlation time of the protein [31];
$C_t = \frac{1}{2k_t}$; k_t is the first-order order rate constant for energy transfer [32];
$A_t = 3/10\ [\cos^2 H_f - \cos^2 H_t]$; $A_{tinf} = 3/10\ [\cos^2 H_f + \cos^2 H_t - 2/3]$;
H_f is the angle between absorption and emission transition moments in the flavins [25]; H_t is the angle between absorption transition moment of A and emission transition moment in D and vice versa [25].

In order to obtain the independent contributions in the total anisotropy the time scales of the depolarizations should be different.
In first approximation it can then be assumed that :

$$1/C_t + 1/C_w \approx 1/C_t$$
$$1/C_t + 1/C_p \approx 1/C_t$$
$$1/C_w + 1/C_p \approx 1/C_w$$

This leads finally to :

$$r(t) = r(0)[(A_w + A_{winf})\ A_t \exp(-t/C_t) + A_{tinf}\ A_w \exp(-t/C_w) + A_{tinf}\ A_{winf} \exp(-t/C_p)] \qquad (11)$$

Equation 11 represents the anisotropy decay at 458 nm excitation.
At 514 nm excitation energy transfer will not be significant and eq. 11 is simplified to :

$$r(t) = r(0)[A_w \exp(-t/C_w) + A_{winf} \exp(-t/C_p)\ ] \qquad (12)$$

The rotational correlation time C_p of the protein can be calculated from an empirical formula [31]:

$$C_p = M\ (\bar{v} + h)\ \eta\ /\ RT \qquad (13)$$

where M is the molecular mass (Da); η the viscosity (centipoise);

$\bar{v}$ the specific volume of the protein (cm^3/g); T the temperature (°K); h the hydration volume of the protein (cm^3/g) ; R the gas constant (J/mole/ °K).

Substituting the appropriate values in eq. 13 a correlation time of 65 ns was found for NADPH-cytochrome P-450 reductase at 10°C.

RESULTS

Fluorescence lifetimes:

The performance of the laser set-up was checked by measuring erythrosine B in water from time to time. The compound showed a monoexponential fluorescence decay curve. The recovered lifetime was 60 ps upon excitation at 458 nm at 10°C and is in good agreement with values published from other laboratories [33].

The fluorescence lifetimes of various samples of NADPH-cytochrome P-450 reductase are collected in Table 1. The experimental curves had to be fitted with four exponentials except for the reconstituted FMN-depleted reductase. The use of only three exponentials led to unacceptable fits. Several flavoproteins studied so far by time-dependent fluorescence techniques all exhibit complex decay curves [31,34,35]. A detailed explanation for this behaviour of protein-bound flavin is difficult to provide, because the multi-exponential decays could be due to different

TABLE 1
Fluorescence decay parameters of different samples of NADPH-cytochrome P-450 reductase. 17 μM of enzyme in 25 mM phosphate buffer, pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 0.1 mM DTT, was measured at 10°C. The data were obtained as described under Materials and Methods.

Sample	λ_{exc} nm	α_1	τ_1 ns	α_2	τ_2 ns	α_3	τ_3 ns	α_4	τ_4 ns	$\bar{\tau}^{a)}$ ns
native	458	0.62	0.08	0.14	0.56	0.09	2.3	0.15	5.3	1.14
native	514	0.50	0.11	0.26	0.69	0.19	2.4	0.05	5.6	0.97
apo [b)]	458	0.39	0.21	0.26	0.83	0.25	2.7	0.09	6.9	1.62
apo [b)]	514	0.40	0.12	0.20	0.80	0.33	2.8	0.06	6.5	1.55
sq anaer [c)]	458	0.63	0.12	0.20	0.61	0.11	2.3	0.05	5.9	0.75
sq aer [d)]	458	0.66	0.09	0.17	0.56	0.10	2.2	0.07	5.5	0.76
rec-FMN [e)]	458	0.24	0.18	0.13	1.74	0.63	5.1	----	---	3.49
free FMN	458	0.11	3.09	0.89	5.11	----	---	----	---	4.88

a) mean lifetime ($\bar{\tau} = \Sigma \alpha_i \tau_i$);
b) FMN-depleted reductase ;
c) semiquinone under anaerobic conditions ;
d) semiquinone under aerobic conditions;
e) FMN-recombined reductase.

effects which are difficult to evaluate quantitatively. Our preparations have been analyzed by various biochemical techniques and found to be homogeneous with respect to cofactor content and impurities by other proteins. Therefore we believe that the complex fluorescence behaviour of the reductase can probably not be ascribed to heterogeneity of the preparations. A structural heterogeneity of reductase molecules, with respect to the flavin environment is another possibility for the observed complex fluorescence behaviour of the protein, but such differences are difficult to observe by any technique, except for laser-induced fluorescence by which a small amount of molecules with variable fluorescence properties can be observed. In such molecules the flavin is still strongly bound but the environment of the isoalloxazine moiety of the prosthetic group is altered in a dynamic fashion. Consequently the interaction between the flavin and the apoprotein is modulated by neighbouring amino acid residues or segments of the protein. Since the environment can be altered continuously, a distribution of microstates should be the result . Therefore a distribution of lifetimes should be a more appropriate model to describe the fluorescence decay kinetics [36]. Since the reductase contains both FMN and FAD, the total fluorescence behaviour of the enzyme becomes even more complex.

Fig. 1 shows an example of the fluorescence decay of native reductase (78 kDa), together with the weighted residuals and the autocorrelation function of the residuals . The latter two curves measure the quality of the fit and the patterns indicate that fitting the experimental curve with four exponentials leads to a perfect fit. The fluorescence lifetimes thus obtained (Table 1) vary from about 0.1 ns to about 5 ns. The shortest lifetime has a contribution of 50-60% to the total fluorescence decay (α-values). The other three lifetimes contribute each to about 10%. The two shortest lifetimes in native reductase are slightly dependent on the excitation wavelengths (458 nm vs. 514 nm), the α-values vary much more (Table 1). The difference in excitation wavelength is more obvious when the values of the mean lifetimes ($\bar{\tau}$) are compared. The effects are more easily observed with the FMN-depleted enzyme. In this sample the shortest lifetimes increase as compared to those of the native enzyme. This is well reflected in the lifetime values. The FMN-depleted sample contains only FAD as prosthetic group. Therefore one is tempted to assign the observed lifetimes to FAD in the native enzyme.

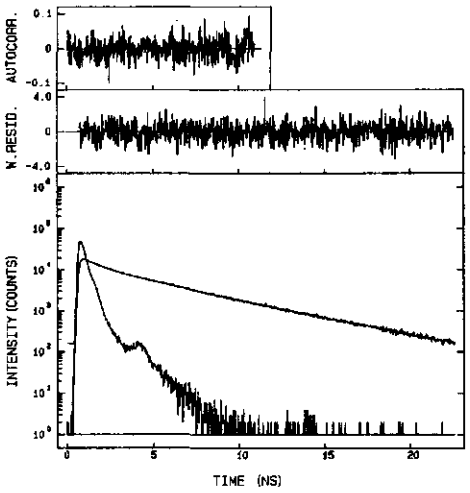

FIGURE 1
Experimental and calculated fluorescence decay curves of native NADPH-cytochrome P-450 reductase (17 μM) in 25 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 0.1 mM DTT at 10°C. The weighted residuals and the autocorrelation function of the residuals are also given. In this particular case the decay parameters are:

α_1=0.62 ± 0.04 τ_1=0.08 ± 0.01 ns
α_2=0.14 ± 0.01 τ_2=0.56 ± 0.05 ns
α_3=0.09 ± 0.01 τ_3=2.26 ± 0.17 ns
α_4=0.15 ± 0.01 τ_4=5.29 ± 0.05 ns

The standard deviations are obtained from the fit.

This interpretation might be correct, but removal of FMN could also influence the conformation of the protein and thereby indirectly the fluorescence properties of bound FAD. That the latter interpretation is more likely follows from the data obtained with native reductase in the one-electron reduced state. These lifetimes resemble much more those of the oxidized native protein than those of the FMN-depleted enzyme, although the mean fluorescence lifetime is decreased.

Reconstitution of the FMN-depleted enzyme by an excess FMN and removal of unbound FMN by column chromatography yields a holoenzyme preparation which cytochrome c activity is almost the same as that of the native protein. The fluorescence properties differ however greatly. This was the only sample tested which could be fitted by three exponentials. The rather long mean fluorescence lifetime indicates that FMN in reconstituted FMN-depleted reductase is bound differently than in native reductase. This interpretation is supported by other data (see below). Therefore the overall activity is probably not a good measure for the judgement of the proper and quantitative binding of FMN to FMN-depleted reductase. At any rate the mean fluorescence lifetime of reconstituted

reductase is smaller than that of free FMN suggesting that FMN interacts with the protein.

In contradiction to published results [37] free FMN exhibits two fluorescence lifetimes (about 10% of a shorter lifetime of 1-3 ns and 90% of a lifetime of 5.1 ns). The contribution of the shorter lifetime component becomes smaller when the buffer is replaced by water alone. In fact it has been found that this is not a unique property of FMN but is also found with various lumiflavin derivatives (unpublished data).
The fluorescence quantum yield of native reductase was determined to be $7.1 \cdot 10^{-4}$ as compared to that of free FMN.

Addition of free FMN to the native reductase leads to an increase of the mean fluorescence lifetime of 1.1 ns to 4.9 ns as expected from the much higher quantum yield of free FMN.

Blumberg et al [9] reported a similar study. They determined the time-dependent fluorescence of free FAD and FMN in the presence and absence of reductase. For the free molecules lifetimes of 1.3 ns were determined for both flavins. These values are in contradiction with our and published values [37,38]. An explanation for these differences is difficult to give but the rather noisy curves of Blumberg et al [9] would certainly yield less accurate values.

Anisotropy measurements:

The results of the time-dependent fluorescence anisotropy measurements are more informative than fluorescence lifetimes and are in addition easier to interprete. The decay parameters are given in Table 2. Fig. 2 represents experimental results together with the fitted data. Most of the experimental data could be fitted with two exponential functions, others had to be fitted with three exponential functions. In some cases this required the fixation of the value of 65 ns for the native protein. Excitation at 514 nm yielded an experimental curve which could be fit with two exponentials. Again using the rotational correlation time of the native protein gave better results than when all parameters were left free during the fitting procedure. The wavelength-dependent experimental curves (Fig.2A,B) clearly demonstrate that excitation at 458 nm creates a component with a short correlation time, which is not observed on excitation at 514 nm. A possible explanation for this observation will be given below.

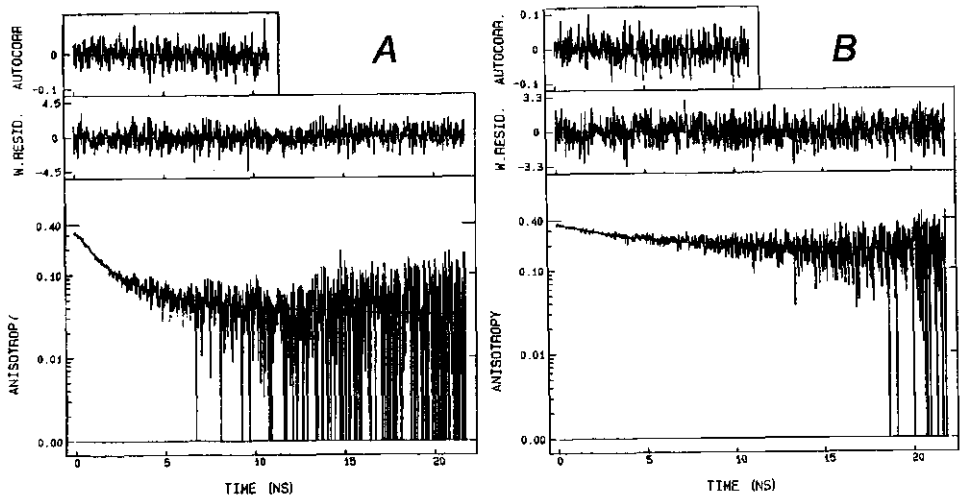

FIGURE 2
Experimental and calculated anisotropy decay curves of native NADPH-cytochrome P-450 reductase (17 μM) in 25 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 0.1 mM DTT at 10°C with excitation at 458 nm (A) or at 514 nm (B). The weighted residuals and the autocorrelation function of the residuals are also given. The decay parameters for fig. 2 A are:

β_1 = 0.23 ± 0.01 ϕ_1 = 0.81 ± 0.04
β_2 = 0.08 ± 0.01 ϕ_2 = 2.76 *)
β_3 = 0.044 ± 0.001 ϕ_3 = 65.2 *)

The decay parameters for fig 2 B are:

β_1 = 0.132 ± 0.002 ϕ_1 = 2.76 ± 0.13
β_2 = 0.229 ± 0.002 ϕ_2 = 65.2*)

*) parameters fixed at fitting.
The standard deviations are obtained from the fit.

TABLE 2
Anisotropy decay parameters of different samples of NADPH-cytochrome P-450 reductase. 17 μM of enzyme in 25 mM phosphate buffer, pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 0.1 mM DTT, was measured at 10°C. The data were obtained as described under Materials and Methods.

Sample	λ_{exc} nm	β_1	ϕ_1 ns	β_2	ϕ_2 ns	β_3	ϕ_3 ns	r(tot)
native	458	0.23	0.81	0.08	2.76*	0.04	65.2*	0.13
native	514	0.13	2.76	0.23	65.2*	----	----	0.29
apo a)	458	0.16	0.88	0.15	20.5	----	----	0.16
apo a)	514	0.16	1.95	0.19	23.8	----	----	0.24
sq anaer b)	458	0.16	2.61	0.18	93.6	----	----	0.26
sq aer c)	458	0.22	1.71	0.12	57.5	----	----	0.20
rec-FMN d)	458	0.34	0.43	0.03	5.0	----	----	0.05
free FMN	458	0.35	0.46	----	----	----	----	0.03

a) FMN-depleted reductase;
b) semiquinone under anaerobic conditions;
c) semiquinone under aerobic conditions;
d) FMN-recombined reductase ;
*) fixed parameters at fitting.

For FMN-depleted reductase, which contained about 2% FMN , showed also wavelength-dependent anisotropy decay profiles. The relatively low value of r(tot) for the FMN-depleted sample (Table 2) is probably due to a less firmly bound FAD, which causes more depolarization than in the native enzyme.

The one-electron reduced reductase showed two correlation times. A rather long one , which is about the same as the rotation correlation time of the protein, and one of about 2 ns. The latter value was also found with all other samples. It is interesting to note that longer correlation times are obtained rather under anaerobic than under aerobic conditions.

Although the semiquinone of the reductase reacts only very slowly with molecular oxygen, the decrease in correlation times could be due to the presence of some oxidized FMN in the reductase sample, leading to rapid depolarization. This is also observed in the total anisotropy (r(tot)) of the semiquinone sample, which decreases from 0.26 to 0.20 if air is admitted to the sample. The correlation times become shorter and the amplitude of the shortest component is increased. Such an observation indicates that the FMN semiquinone becomes partially oxidized and energy transfer occurs. Reconstitution of FMN-depleted reductase yields correlation times which are very different from those observed with the native protein, although the reconstituted sample exhibited an activity of about 95% of that of the native protein. The longer correlation time is about 10 times as long as that of free FMN. From this it could be concluded that FMN is rather loosely bound to the protein. The same conclusion could also be drawn from the corresponding amplitudes (β-values, Table 2). The presence of free FMN is unlikely because the sample of the reconstituted protein was chromatographed prior to the measurements , removing unbound FMN from the protein. Therefore we conclude that FMN in reconstituted reductase is bound very differently from that in native protein.

From the results above it follows that NADPH-cytochrome P-450 reductase shows two to three correlation times. The value of ~ 65 ns represents the rotational correlation time of the native protein (see Methods). The shorter value(s) is (are) ascribed to the mobility of the flavins in the protein. It cannot be excluded that this mobility is due to a domain or fragment of the protein to which the flavin is strongly

bound .

It is also of importance to note that the presence of the shortest correlation time is dependent on the wavelength of excitation (Fig.2). Excitation at longer wavelengths eliminates this component from the decay (cf. Fig.2). This observation suggests that energy transfer occurs between the two flavins. This suggestion will be worked out in more detail below.

Steady state fluorescence anisotropy:

The above described time-dependent anisotropy and fluorescence lifetime data (Table 1 and 2) strongly suggest that energy transfer occurs between the two flavins in the reductase. The steady state wavelength-dependent anisotropy of native and FMN-depleted NADPH-cytochrome P-450 reductase is shown in Fig.3A. Both preparations exhibit the expected wavelength-dependent anisotropy for the two lowest transitions of the flavin molecule, except that the anisotropy for the native enzyme is lower in the 400-500 nm region than that of the FMN-depleted form. The final values at 520 nm are the same for both preparations and agree well with the initial values (± 0.4) observed by the time-dependent anisotropy measurements.

As a model system for the NADPH-cytochrome P-450 reductase we investigated N(3)-methyllumiflavin and tri- and hexa-methylene-bis-10,10'-(3,7,8-trimethylisoalloxazine) immobilized in polymethyl metacrylate. The fluorescence lifetime and time-dependent anisotropy properties of these compounds have already been determined earlier [24]. The steady state anisotropy curves of these compounds is shown in Fig.3B. The steady state anisotropy of the monomeric compound across the first transition is about 0.38. The corresponding values for the dimeric flavin molecules are lower and differ among them. Remarkable however is that the steady state anisotropy of the dimeric flavins increases at wavelengths longer than 480 nm and reaches that of the monomeric flavin at about 510 nm.

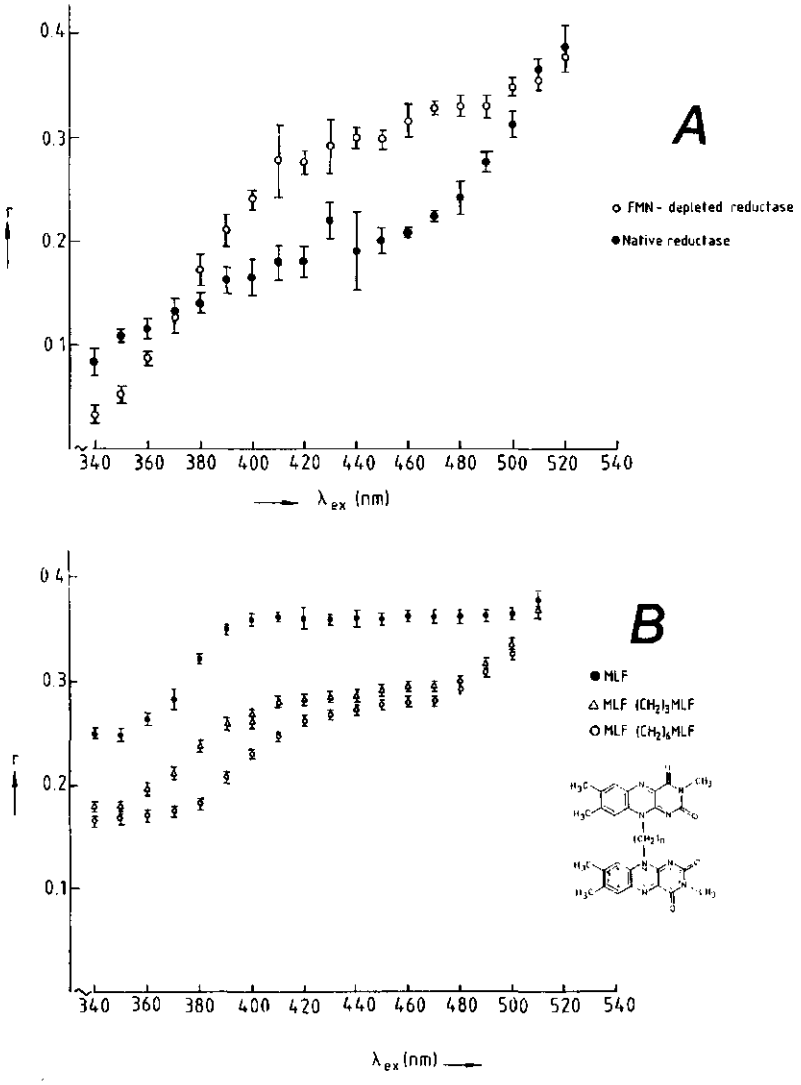

FIGURE 3
Steady state fluorescence anisotropy (r) of native and FMN-depleted NADPH-cytochrome P-450 reductase (A) and of methylluмiflavin, MLF-$(CH_2)_3$-MLF in polymethyl methacrylate and MLF-$(CH_2)_6$-MLF (B) as function of the excitation wavelength (λ_{ex}). The buffer used was : 25 mM potassium phosphate , pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 0.1 mM DTT. The measuring temperature was 10°C.

DISCUSSION

The decay of the fluorescence emission is composed of multiple relaxation as manifested by calculational methods (Table 1). We found that the experimental data could best be fitted by four and in one case by three exponential decays. These interpretations should be considered as a minimum model. Since the observed heterogeneity of the fluorescence emission of the complex protein could be caused by different mechanisms it is difficult to assign the observed relaxations to a particular effect. As mentioned above the preparations were analyzed by various biochemical techniques with respect to protein homogeneity and cofactor content. The preparations were found to be of high quality. Therefore we believe that these factors contribute probably only little to the observed fluorescence heterogeneity. It is now well accepted that biomolecules are not static molecules but undergo dynamic structural changes in solution. These dynamic changes consist of spatial displacements of amino acid residues in the immediate neighbourhood of the isoalloxazine moiety of the prosthetic groups or of structural fluctuations of segments or domains of the protein. This probably causes the fluorescence to appear heterogeneous. Hence a distribution of lifetimes is observed rather than lifetimes attributable to distinct states of the structure of the protein. From this it is obvious that one must be cautious with a detailed interpretation of the experimental data. Despite this handicap it is still possible to extract some interesting information from time-dependent fluorescence data, especially from anisotropy data.

As already mentioned under Results the anisotropy data of native reductase could best be fitted by three exponential decays. These required the fixation of some values by trial and error procedures (Table 2). This procedure was not required for all data collected to obtain an acceptable fit. The correlation time of 2.7 ns for native reductase, fixed in one particular experiment only (see Table 2), was also obtained for other samples when the values were left free during the fitting procedure. In fact the observation of this value in most other samples prompted us to introduce it as a fixed parameter into the fitting of entry one in Table 2. Only in this way consistent fits of the experimental data could be obtained. An interesting point is the wavelength-dependence of the correlation times. Excitation of native reductase at

514 nm instead at 458 nm yields two correlation times and the total anisotropy increases. Similar effects were observed with FMN-depleted reductase. The lower steady state anisotropy values obtained from this sample suggest that the binding interaction between FAD and the protein is altered or some structural changes occur by the preparation of the FMN-depleted reductase. The sample of the reductase in the semiquinone state under anaerobic conditions also shows two correlation times, but the longest component is increased to 94 ns as compared to those of all other samples.
Also the total anisotropy is increased. The observed value is very similar to those observed from native and FMN-depleted reductase excited at 514 nm, but dissimilar to those observed on excitation at 458 nm. Since the value of 65 ns represents the rotational correlation time of completely immobilized, protein-bound flavin in native reductase, the value of 94 ns indicates that the molecular mass of the semiquinone of reductase is increased, probably by formation of dimeric protein molecules or even higher order quaternary structures.

When the air-stable semiquinone was prepared under aerobic conditions lower steady-state anisotropy values were observed (Table 2). This decrease must be ascribed to the presence of a few percent of fully oxidized reductase in the sample. This was ascertained by spectrophotometric measurements at the begin and end of the fluorescence experiments and it was found that under anaerobic conditions 100% of semiquinone, whereas under aerobic conditions about 90-95% of semiquinone was present in the sample.

Reconstitution of FMN-depleted reductase yielded very low steady-state anisotropy values (Table 2), although the samples used were gel chromatographed immediately prior to the experiments. In addition activity measurements indicated that the samples were 90-95% active as compared to native protein. Therefore we must conclude that FMN in reconstituted FMN-depleted reductase is bound differently than in the native protein. This suggests that the activity alone is not a reliable parameter to conclude that the binding of FMN in native and reconstituted FMN-depleted reductase is the same. Nevertheless the correlation time of 5.0 ns of FMN-depleted reductase, although about one order of magnitude smaller than that observed for native protein but one order of magnitude larger than that of free FMN, indicates that

FMN is bound to the reductase.

The wavelength-dependent time-resolved and static anisotropy data of native and FMN-depleted reductase (Table 2, Fig. 3) suggest that energy transfer occurs between the two flavins in the protein. This suggestion is supported by the data obtained from the air-stable semiquinone of the reductase under anaerobic and aerobic conditions. Under anaerobic conditions, where only one flavin (i.e. FAD) is fluorescent, the total anisotropy is about the same as that observed from native and FMN-depleted reductase on excitation at 514 nm. The possible occurrence of energy transfer between the flavins in reductase is more obvious from the steady state anisotropy data (Fig. 3A) showing that the anisotropy in the excitation region 420-480 nm is smaller for native than for FMN-depleted reductase. To corroborate the idea of energy transfer in reductase we investigated model systems in the immobilized state mimicking our protein system. The steady state anisotropy of the monomeric N(3)-methyllumiflavin is larger than those of the dimeric flavins (Fig. 3B). Similar observations were made with native and FMN-depleted reductase. A common observation in both sets of experiments is that the steady state anisotropy at about 514 nm becomes equal (Fig. 3A, B). Also the time-dependent anisotropies of the model systems (cf. Fig. 3 in [24]) and the reductase samples show great similarities, i.e. the dimeric flavins and native reductase exhibit a fast initial decay of the anisotropy whereas FMN-depleted reductase and monomeric flavin do not. Since it has been demonstrated that energy transfer occurs in the model systems [24] we can now state that this is also the case in native reductase based on the great similarities of both sets of data.

The larger anisotropy difference between native and FMN-depleted reductase than between lumiflavin and its dimers can be explained by the higher value of the orientation factor κ^2 (see eq. 6) in native reductase, i.e. the transition moments possess an orientation more favorable for energy transfer.

The observation that no energy transfer is observed at λ_{exc} >480 nm is known as "red edge failure" of energy transfer [39]. This phenomenon implies that at red edge excitation those molecules will be excited which have maximal interaction energy in the ground state and minimal interaction energy in the excited state with the environment . If the dipole-reorientation time (τ_r) is longer than the fluorescence lifetime (τ_f)

, there is no redistribution of the energy states by thermal relaxation and no energy transfer is possible. Energy transfer is only possible if the energy difference between the S_0 and the S_1 states in the acceptor molecule is equal to or smaller than the one in the donor molecule . If we represent the donor and acceptor S_0-S_1 energy difference as a distribution the proces is easily understood. The probability (P) that donor and acceptor have an almost equal energy difference is $P(\Delta E_A) \cdot P(\Delta E_D)$. This probability becomes smaller when the system is excited at the red edge of the absorption band. If $\tau_r << \tau_f$ the system wil relax back to the more favorable state, having a high probability . The probability for an equal energy difference between S_0 and S_1 of donor and acceptor is then also high and this favors energy transfer.

Using the formalism described under Materials and Methods the rate constant of energy transfer (k_t) and the angle between the optical transition moments of FAD and FMN can be obtained from anisotropy measurements at 458 nm. The results obtained at 514 nm, on the other hand, give the correlation time of restricted cone motion of the flavins and the angular amplitude of the cone motion. To calculate these data some assumptions must be made , which are : a) the fluorescence quantum yield of FAD is similar to that of FMN (this seems a reasonable assumption because otherwise we would not be able to observe energy transfer) and b) the fluorescence lifetime of the donor and acceptor are also about the same (this is supported by the data collected in Table 1, i.e. 1.1 ns for native and 1.6 ns for FMN-depleted reductase). In Table 3 the relevant energy transfer parameters have been collected.

Dale and Eisinger [40] described four models for the "wobbling" in a cone and critically evaluated the orientation factor and dynamic depolarization factors for various donor-acceptor systems. We will consider only the model of diffusion in the volume of a cone, because this seems

TABLE 3
Parameters relevant for interflavin energy transfer.

Fluorescence quantum yield (Q_f)	: $7.1 \cdot 10^{-4}$ (native reductase) $1.4 \cdot 10^{-3}$ (FMN-depleted reductase)
Spectral overlap integral (J)	: $4.64.10^{-15}$ cm^3/mole
Refractive index medium (n)	: 1.4
Rate constant for energy transfer (k_t)	: $0.62 \cdot 10^9$ s^{-1}
Fluorescence lifetime donor (τ_D)	: 1.1 - 1.6 ns

TABLE 4
Top angles (θ) of the cone of "wobbling" for different reductase samples*)

Sample	λ_{exc} (nm)	θ (°)
Native reductase	514	31
FMN-depleted reductase	458	44
Semiquinone anaerobic	458	36

*) determined as described by Lipari and Szabo [30].

TABLE 5
Values of the orientation factor κ^2 and critical transfer distance R_0 for different top angles of the cone of "wobbling" and for different models*)

Model	θ (°)	κ^2	R_0 (nm)	Model	θ (°)	κ^2	R_0 (nm)
1	31	2.9	1.14	1	44	2.2	1.22
2	31	0.14	0.69	2	44	0.27	0.86
3	31	0.28	0.77	3	44	0.50	0.95
4	31	0.78	0.92	4	44	0.64	0.99

*) Models 1-4 refer to Figures 17,38,31,23 respectively of Dale and Eisinger [40].

the most realistic model for our systems where FAD and FMN are considered to fluctuate independently of each other.

The top angles of the cone were evaluated as described by Lipari and Szabo [30]. The results are shown in Table 4. A value between 31 and 45 degrees is probably an appropriate value for the top angle. The other needed parameters were obtained as follows.

From the pre-exponential factor of the shortest anisotropy component (A_t or β_1) of the native enzyme excited at 458 nm, the angle between the optical transition moments in FAD and FMN can be obtained. The angle H_t was found to be 67 degrees. The angle H_f between the absorption and the emission dipoles in reductase in the absence of the donor (acceptor), i.e. FMN-depleted reductase, was obtained from r(0) and found to be 17 degrees. In order to cover other angular boundaries the limits of the angles can be set to 0 and 90 degrees. Considering these values and the models of Dale and Eisinger (1975) pertinent to this case (Figs. 17,23,31 and 38 of ref.[40]) upper and lower values of the orientation factor can be calculated for the two values of the top angle of the cone (31 and 45 degrees). In addition, from the data of Table 3 upper and lower limits of the critical transfer distance can be calculated. All these data have been collected in Table 5. It is straightforward now to evaluate the boundaries of the actual distance between FAD and FMN. For a lifetime of

the donor of 1.1 ns and using eq. 4 this gives a distance between the FAD and the FMN of 0.73-1.21 nm and a distance of 0.86-1.22 nm for a lifetime of the donor of 1.6 ns. This seems a reasonable distance between the centers of the two flavins for transport of reduction equivalents and it also implies that there is no interflavin contact in the NADPH-cytochrome P-450 reductase.

Although many dimeric flavoproteins are known there is no evidence that electrons use, like in NADPH-cytochrome P-450 reductase, one flavin as the entry port and the other as the exit port. Recently the three-dimensional structure of flavocytochrome b_2 from bakers yeast at 0.24 nm resolution became available [41,42]. This flavoprotein also contains a heme group as electron carrier. The enzyme exhibits a similar electron transfer mechanism as NADPH-cytochrome P-450 reductase. The crystallographic study shows that the two prosthetic groups are separated by 1.6 nm. The distance between FAD and FMN in NADPH-cytochrome P-450 reductase is calculated to be < 1.6 nm from fluorometric data. This suggests that reasonable distances can be calculated from fluorometric data, although some assumptions had to be made.

From the above it is now also possible to assign the different correlation times (Table 2) more specifically. The longest correlation time corresponds quite well with the expected rotational correlation time of a protein of 78 kDa.
The correlation time of about 2.7 ns is probably due to internal motion of segments of the protein. The correlation time of about 1 ns is only observed upon excitation of native protein at 458 nm. This strongly suggests that this contribution to the anisotropy is due to energy transfer.

In conclusion it can be stated that time-dependent and static fluorescence measurements yield useful information regarding the structure of proteins and contribute to a better understanding of the mechanism of the protein.

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CHAPTER 4

RECOMBINATION OF FMN-DEPLETED NADPH-CYTOCHROME P-450 REDUCTASE WITH DIFFERENT FLAVINS

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SUMMARY

FMN-depleted NADPH-cytochrome P-450 reductase was reconstituted with different flavin compounds. The association rate constant was determined by activity measurements and stopped flow techniques. All flavins tested gave activities in the range of 70-100% as compared with that of native enzyme. Riboflavin 3',5'-biphophate does not bind to the enzyme, indicating that the extra phosphate at the 3' position of the ribityl side chain of the prosthetic group prevents binding either due to the extra charge or the bulkiness of the group. This contrast the structural variations allowed in the benzene subnucleus of the prosthetic group. Even an additional benzene ring appears not to hamper the interaction with the protein. This observation suggests that FMN in the reductase is possibly located on the surface of the protein, in analogy with flavodoxins. The association rate constants as measured by stopped flow techniques show only little variation for the different flavins, except for riboflavin.

INTRODUCTION

NADPH-cytochrome P-450 reductase is a membrane bound flavoprotein present particularly in liver microsomes [1-5]. It is a part of the mixed function oxidation system (MFO-system) and transports reduction equivalents from NADPH to cytochrome P-450. This multi-enzyme system plays an essential role in the detoxification process of xenobiotica, activation of procarcinogens, the steroid- and fatty acid metabolism etc. [6-11]. The enzyme contains two flavins , FMN (flavin mononucleotide) and FAD

(flavin adenine dinucleotide) in equimolar ratio. The electrons enter on the FAD site and leave at the FMN site to cytochrome P-450 [4,12-14].

In this paper we have addressed the question whether or not FMN can be exchanged with other flavins and how the activity of the enzyme is affected by this procedure. The association reaction between FMN-depleted NADPH-cytochrome P-450 reductase and various flavins was studied by different methods : UV absorption, fluorescence and enzymic activity.

First we measured the quenching of the fluorescence of the free flavin by the FMN-depleted reductase. Second we measured the cytochrome c reduction by the native reductase during recombination of the FMN-depleted reductase and the free flavin. However, we measured the reduction of cytochrome c. This time the obtained k_2' value describes not only the association but in fact the overall reaction : binding of the free flavin, binding of cytochrome c and NADPH, and the reduction. If the binding of cytochrome c + NADPH and the reduction are very fast compared to the binding of the flavin the two methods can be compared. The K_m values of the cytochrome c and the NADPH are known (resp. 10 and 5 μM [15,16]). We measured the Km-values of the free flavins. Both methods are employed as well with conventional fluorescence and absorption instruments as with a stopped flow apparatus with fluorescence and absorption detection.

MATERIALS AND METHODS

Isolation:

NADPH-cytochrome P-450 reductase was isolated from rabbit and rat livers as described previously [3] with some minor modifications. Microsomes were solubilized with lubrol and sodium desoxycholate. The solubilized microsomes were applied on a DEAE cellulose column. After washing with 0.1 M KCl in the DEAE equilibration buffer (25 mM Tris/HCl pH 7.7, containing 10% glycerol, 0.2% desoxycholate, 0.1% lubrol, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF) the reductase was eluted with 0.4 M KCl in the same buffer. The DEAE-eluate was applied to a 2'5'-ADP Sepharose column (Pharmacia). After a wash procedure the yellow NADPH-cytochrome P-450 reductase was eluted with 2'3'-AMP. The sample was concentrated by ultrafiltration and stored at -80°C untill used. The final reductase sample was pure as shown by SDS-

polyacrylamide gelelectrophoresis according to Laemmli [17]. Specific activity of the pure reductase varied from 40-50 u/mg. Activity was measured at 25°C by measuring the cytochrome c reduction at 550 nm ($\Delta\epsilon$ =21.0 $mM^{-1}cm^{-1}$) in 300 mM potassium phosphate pH 7.7. Protein concentration was determined by the method of Lowry [18], modified by Peterson [19] for samples containing detergents.

Procedure for preparation of FMN-depleted reductase:

An aliquot of a stock solution of reductase was diluted into 400 ml "apo-buffer" (100 mM Tris/HCl pH 8.4 (4° C), containing 20% glycerol, 2 M KBr, 0.1 mM EDTA, 1.0 mM DTT, 0.1 mM PMSF). The solution was concentrated at 4°C with a 400 ml Amicon apparatus with an YM-30 filter. The concentrate was diluted again in the same buffer to 400 ml and the procedure was repeated several times. After the final concentration step the KBr was removed by gelfiltration over Biogel 6-PDG in 25 mM potassium phosphate , pH 7.7, containing 10% glycerol, 0.1% lubrol, 0.1 mM DTT, 0.1 mM EDTA.

Reconstitution:

Reconstitution of the FMN-depleted reductase (apo) with the modified flavins was studied under pseudo-first-order kinetics by different techniques.

The kinetics was studied by following the quenching of the fluorescence of free flavin by FMN-depleted reductase :

APO-REDUCTASE + FLAVIN ⇆ HOLO-REDUCTASE
fluorescent non-fluorescent

If [APO] >> [Flavin] we have pseudo-first-order kinetics , then $v = -d[Flavin]/dt = k_{ps}[Flavin]$ in which $k_{ps} = k_2[APO]$; k_2 is the association rate constant and is a measure of how fast the flavin recombines with the apo-reductase.

In other experiments we measured the cytochrome c reduction by the holo-reductase during recombination of the FMN-depleted reductase and free flavin:

APO-REDUCTASE + FLAVIN ⇆ HOLO-REDUCTASE + CYT C(OX) + NADPH ⇆
HOLO-REDUCTASE + NADP + CYT C(RED)

If [Flavin] >> [APO] we have again pseudo-first-order conditions for the recombination reaction. However, we measure the reduction of cytochrome c. This time the obtained k_2' value describes not only the

association but in fact the overall reaction : binding of free flavin, binding of cytochrome c + NADPH and the reduction of cytochrome c. If the binding of cytochrome c and NADPH, and the reduction of cytochrome c are very fast compared to the binding of the flavin the two methods can be compared. The K_m values of cytochrome c and NADPH are known (10 and 5 μM, respectively [15,16]). We also measured the K_m values of the free flavins.

Both methods, conventional fluorescence and absorption measurements, as well as stopped flow fluorescence and absorption techniques were used.

Experiments were performed as follows :

(A) Reconstitution was followed by restoring the cytochrome c reduction ability of the enzyme. Cytochrome c (53 μM) was incubated at 25°C with buffer (300 mM potassium phosphate, pH 7.7), flavin (varying concentrations) and NADPH (66 μM). At t=0 cytochrome c reduction was started by adding FMN-depleted enzyme (1.6 nM final concentration). The absorbance at 550 nm (λ_{max} reduced cytochrome c) was followed in time until $\Delta A550/\Delta t$ was constant. Corrections were made for the rest activity of the FMN-depleted reductase.

(B) Reconstitution was followed by quenching of flavin fluorescence by FMN-depleted enzyme using an Aminco spectrofluorometer (λ_{exc}= 445 nm, λ_{em}=525 nm). Flavin (72.8 nM final concentration) was incubated in 300 mM potassium phosphate, pH 7.7, at 25°C. At t=0 FMN-depleted enzyme (389 nM final concentration) was added and the fluorescence of the flavin was measured until a plateau value was reached.

(C) The same as in (A) but the reaction was performed with a stopped flow apparatus. FMN-depleted reductase (49 nM in 300 mM potassium phosphate, pH 7.7) was rapidly mixed in a 1:1 ratio with a solution containing 400 μM cytochrome c, 200 μM NADPH, varying amounts of flavin in 300 mM potassium phosphate, pH 7.7, at 25°C. The increase of the absorbance at 550 nm was recorded.

(D) The same as in (B) but the reaction was performed with a stopped flow apparatus. FMN-depleted reductase (varying concentrations in 300 mM potassium phosphate, pH 7.7) was rapidly mixed in a 1:1 ratio with

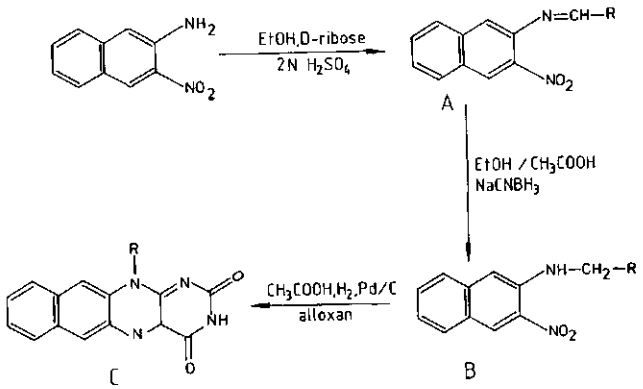
a flavin solution (about 200 nM in 300 mM potassium phosphate, pH 7.7). The decrease of the fluorescence (λ_{exc}= 450 nm, λ_{em}= 530 nm, cut off filter) was recorded.

Equipment:

The stopped flow apparatus (Dept. of Biophysical Chemistry, Katholic University of Nijmegen, The Netherlands) has a cuvet volume of 67 µl and a dead time of 3-4 msec.

Synthesis of modified flavins:

The modified flavins were virtually synthesized according to published procedures [20] with some modifications as examplified by the following synthesis (cf.Scheme 1) :



SCHEME 1
Synthesis of modified flavins.

3-Nitro-naphthalidino-D-ribofuranoside (A) :

0.8 g (4.26 mmole) of 2-amino-3-nitronaphtalene and 0.7 g (4.67 mmole) of D-ribose were dissolved under heating in 40 ml abs. alcohol. To the solution cooled to 25°C were added 3 drops of 2 N H_2SO_4 and the solution let stand overnight at 4°C. The formed crystals were then filtered off and washed with little abs. alcohol, followed by ether; yield 1.2 g (88.2%). For elemental analysis some of the compound was twice recrystallized from CH_3CN. Melting point 186-187°C. $C_{15}H_{16}N_2O_6$ (320.30) calculated : C 56.25, H 5.04, N 8.75% ; found C 56.5, H 5.0, N 8.6%.

N-(D-ribityl)-3-nitronaphthalidine (B) :

A solution of the aminal A (0.8 g, 2.5 mmole) in 20 ml of a mixture of alcohol/glacial acetic acid (1:1) was treated with 1.0 g of $NaCNBH_3$, added in three portions, at room temperature for several hours. The reaction was followed with TLC using precoated thin layer plates, Silicagel IB2, Bakerflex, and CH_3CN as a mobile phase. The time (4-5 h) needed for the reduction of the aminal can be shortened by warming the reaction mixture to 50-60°C. After completion of the reaction the reaction mixture was evaporated to dryness. The residue was suspended in 40 ml H_2O, the precipitate filtered off, washed with water and air-dried. Yield 0.75 g (93%). Crystallization from CH_3CN gave an analytical pure compound : m.p. 175-177°C, $C_{15}H_{18}N_2O_6$ (322.32) calculated : C 55.90, H 5.63, N 8.69% ; found : C 55.9, H 5.6, N 8.6%.

The above described procedure deviates from the usual method to prepare flavin derivatives from aromatic ortho-nitro compounds in that the aminal is catalytically reduced to the corresponding ortho-diamine and directly condensed with alloxan. Since the ortho-diamines are extremely oxygensensitive and additional handling of the solution is necessary before the condensation reaction, the yield of the flavin is far from optimal.

1-D-Ribityl-naphtho[2,3-g]pteridine-2,4(3H,12H)-dione (C) :

0.40 g (0.82 mmole) of compound B was dissolved in 200 ml glacial acetic acid and catalytically ($Pd/C,H_2$) reduced to the corresponding diamine at roomtemperature and atmospheric pressure. The solution thus obtained was filtered anaerobically into an Erlenmeyer flask containing 0.16 g (1 mmole) of alloxan hydrate and 0.06 g (1 mmole) of boric acid. The solution was then stirred overnight at room temperature under anaerobic conditions (N_2). The reaction mixture was evaporated almost to dryness, the residue suspended in 50 ml H_2O, the precipitate filtered off, washed with H_2O and air dried. Yield: 0.4 g (65.6%). For analytical purposes 0.1 g was tetraacetylated by a published procedure [21] and purified by column chromatography (Kieselgel Mallinckrodt; 100 mesh, Serva, Heidelberg) using $CH_2Cl_2/CHCl_3$ (1:1) mixture as mobile phase. M.p. 246-249°C, $C_{27}H_{26}N_4O_{10}.\frac{1}{2}\ H_2O$ (575.52) ; calculated : C 56.30 , H 4.69, N 9.73, O 29.19% ; found : C 56.4, H 4.7, N 9.8, O 28.8 %.

The isomeric compound (2) (cf. Fig.1) was synthesized in the same way, with the following modification :
2.0 g (1.06 mmole) of 3-amino-4-nitronaphthalene and 2.0 g (1.33 mmole) of D-ribose were dissolved in 150 ml abs. ethanol under heating. To the solution cooled to room temperature were added four drops of 2 N H_2SO_4 and the solution led stand overnight at 4°C. The crystalline product was filtered off, washed with ethanol and air-dried. TLC showed that a major portion of the starting material co-crystallized with the product. The starting material was suspended in CHCl3 and stirred at roomtemperature for several hours. This removed most of the starting material from the product. After filtration the CHCl3 solution was evaporated and the residue dissolved in the mother liquor of the reaction. This solution was again refrigerated overnight and the precipitate collected. The procedure was repeated three times yielding a total of 3.1 g (91.2%) of 4-nitro-naphthalidino-D-ribofuranoside. Recrystallization from CH_3CN yielded an analytical pure compound. M.p.202-204°C. $C_{15}H_{16}N_2O_6$ (320.30) calcd: C 52.25, H 5.04, N 8.75% ; found: C 56.1, H 5.1, N 8.5%.

2.0 g (6.25 mmole) of the aminal was suspended in 50 ml of a mixture of glacial acetic acid/EtOH (1:1), 2.0 g $NaCNBH_3$ added and the mixture stirred for 1 day at room temperature. The reaction was followed by TLC. If necessary more NaCNBH3 was added and the temperature rised to about 50°C. After completion of the reaction the solvent was evaporated, the residue suspended in H_2O and the precipitate filtered off and air-dried. Yield 1.8 g (90%). The product was recrystallized from CH_3CN. M.p. 187-189°C. $C_{15}H_{18}N_2O_6$ (322.32); calcd : C 55.9, H 5.63, N 8.69% ; found : C 55.9, H 5.6, N 8.7%.

The final product 1-D-ribitylnaphto[3,4-g]pteridine-2,4(3H,12H)-dione was obtained in the same way as described for the isomeric compound with a yield of 66.6%. The tetraacetyl derivatives gave the following analytical results: m.p. 255-258°C. $C_{27}H_{26}N_4O_{10}.½\ H_2O$ (575.52); calcd: C 56.35, H 4.69, N 9.73, O 29.19 %; found : C 56.4, H 4.6, N 9.8, O 29.0 %.

The synthesis and purification of riboflavin 3,5-biphosphate was described elsewhere [22]. The syntheses of 2-thio-FMN, 3,4-dihydro-FMN and 3,4-dihydro-iso-FMN will be published elsewhere (F.Müller and J.Lee, unpublished results). FMN was purified as described before [23]. Riboflavin and FMN were obtained from Sigma.

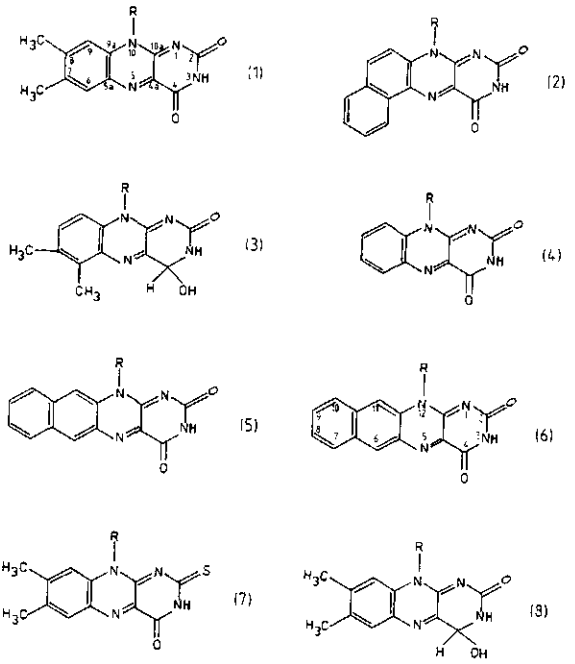


FIGURE 1
Structures of different flavins used in this study.

(1) *FMN* : R = $CH_2(CHOH)_3-CH_2-O-P$
Riboflavin 3'5' biphosphate : R = $CH_2CHOH-CHOP-CHOH-CH_2-O-P$
Riboflavin : R = $CH_2(CHOH)_3-CH_2OH$
(2) 6,7 ϕ-FMN
(3) 3,4-dihydro-6,7-dimethyl-10-ribitylisoalloxazine 5'-phosphate
(4) 10-ribitylisoalloxazine 5'-phosphate
(5) 7,8 ϕ-FMN
(6) 7,8 ϕ-Riboflavin
(7) 2-thio-FMN
(8) 3,4-dihydro-FMN

TABLE 1
The relative activity of FMN-depleted NADPH-cytochrome P-450 reductase recombined with different flavins.

Flavin	% Activity[1)]
FMN	100
6,7 ϕ-FMN	110
Riboflavin	72
3,4-dihydro-FMN	91
2-thio-FMN	82
3,4-dihydro-6,7-dimethyl-10-ribitylisoalloxazine 5'-phosphate	89
10-ribitylisoalloxazine 5'-phosphate	71
7,8 ϕ-FMN	32
7,8 ϕ-Riboflavin	94
Riboflavin 3',5'-biphosphate	11

1) % Activity = $(U_+ - U_0) / (U_{+FMN} - U_0) * 100\%$
U_+ : activity of apo-reductase in the presence of flavin
U_0 : activity of apo-reductase in the absence of flavin
U_{+FMN} : activity of apo-reductase in the presence of FMN

RESULTS

Fig. 1 shows the different flavins used in this study.

In Table 1 the activities of the FMN-depleted enzyme incubated with different modified flavins are summarized. The percentual activity of the different flavins shows that FMN-depleted reductase reconstituted with 6,7 ϕ-FMN is even more active than native reductase. The other flavins, except riboflavin 3'5'-biphosphate and 7,8 ϕ-FMN, show an activity of about 70-95% after reconstitution with FMN-depleted reductase. Riboflavin 3'5'-biphosphate shows after recombination with the FMN-depleted reductase low activity. An extra phosphate group on the 3' position of the ribityl chain is probably disadvantageous for the binding and the reconstitution of the activity. 7,8 ϕ-FMN shows a rather low activity probably because this flavin sample contains impurities (7,8 ϕ-FMN could not be purified by DEAE column chromatography).

Reconstitution of FMN-depleted reductase with FMN and 6,7 ϕ-FMN was performed as described in Materials and Methods (A). Because we measured under conditions of pseudo-first-order kinetics (i.e. [flavin] >> [apo]), $v = -d[apo] / dt = k_{ps} [apo]$ in which $k_{ps} = k_2 [flavin]$. Integration gives $\ln([apo]_t / [apo]_0) = -k_{ps} t$. The potential activity, A_{pot}, of the FMN-depleted sample is a measure of the concentration of FMN-depleted reductase and was calculated by :

$$A_{pot} (\%) = 100\% - A_{t,corr} (\%)$$

$$A_{t,corr} (\%) = (A_t - A_0) / (A_\infty - A_0) * 100\%$$

A_t : activity at time t;

A_0 : activity at time t=0 (before addition of flavin);

A_∞ : activity at time t=∞ after addition of flavin.

In Fig. 2 the results are plotted in a semilogarithmic way for several concentrations of FMN. The slope gives the pseudo-first-order rate constant k_{ps}. Fig. 3 gives the plot of k_{ps} versus FMN-concentration and the slope of this plot gives the second order association rate constant k_2'.

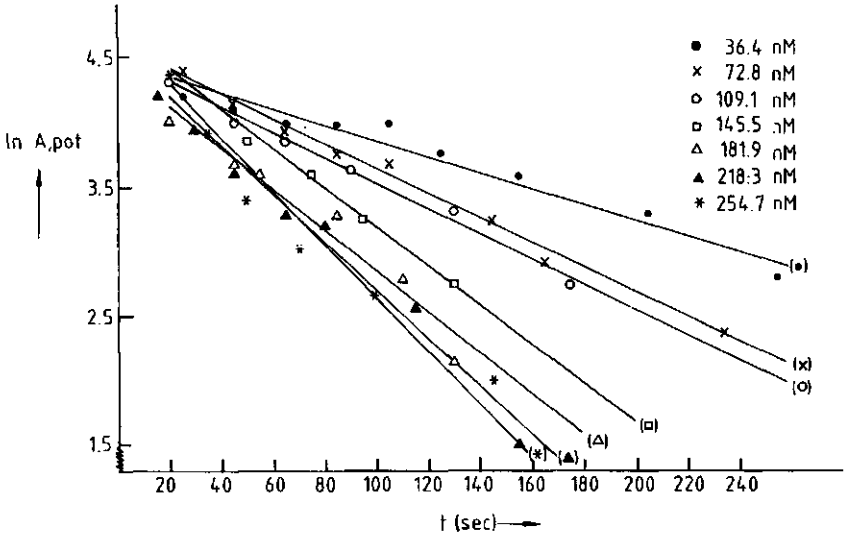
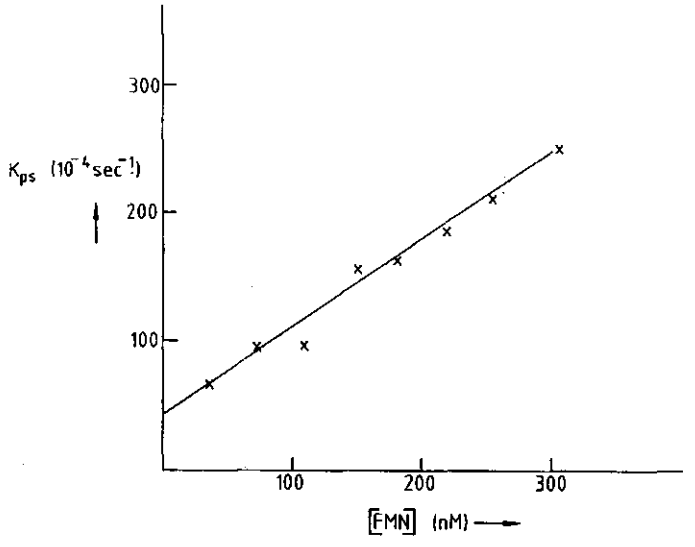


FIGURE 2
Semilogaritmic plot of ln A_{pot} (see text) against time t for several concentrations of FMN. A solution of cytochrome c , NADPH and flavin in 300 mM potassium phosphate buffer, pH 7.7, was mixed with FMN-depleted reductase in 300 mM potassium phosphate pH 7.7. The activity was measured by following the cytochrome c reduction at 550 nm in time. This procedure was repeated for different flavin concentrations at 25°C. The concentration of FMN-depleted reductase was constant (1.6 nM).

FIGURE 3
Plot of k_{ps} (pseudo-first-order rate constant) obtained from Fig. 2 against FMN-concentration. For further explanation see legend to Fig. 2.



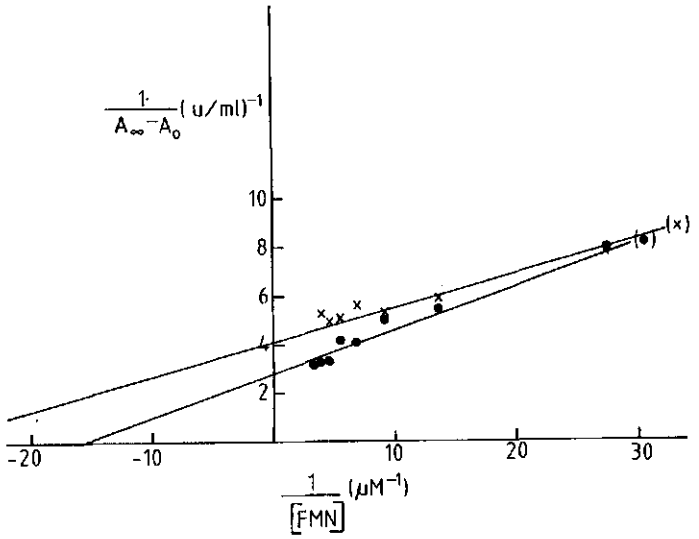


FIGURE 4
Plot of $1/(A_\infty - A_0)$ (see text) against 1/[FMN]. X-intercept = $1/K_m$
For further explanation see legend to Fig. 2.

The K_m value of the two flavins can be calculated by plotting $1/(A_\infty - A_0)$ versus 1/[flavin]. Fig. 4 gives this plot for FMN. This K_m value is in fact not the real K_m because we measured not directly the association of the flavin but the reduction of cytochrome c as a consequence of the incorporation of flavin into the FMN-depleted reductase. A more reliable value for the affinity is K_m/% Activity. In Table 2 the results of three different apo-samples are summarized. We can see that there is almost no difference in the association rate constant k_2' of FMN and of 6,7 ϕ-FMN. The affinity of FMN is only slightly greater than that of 6,7 ϕ-FMN. We can conclude from these data that the extra phenyl group at the 6,7 position of the isoalloxazine ring is of no concern for

TABLE 2
Recombination parameters of FMN and 6,7 ϕ-FMN with three different FMN-depleted reductase preparations as measured with cytochrome c reduction.

Sample	[apo] nM	FAD/FMN	A_- (U/ml)	A_+ (U/ml)	FMN $k_2'(10^4$ $M^{-1}sec^{-1})$	FMN K_m (nM)	6,7 ϕ-FMN $k_2'(10^4$ $M^{-1}sec^{-1})$	6,7 ϕ-FMN K_m (nM)
Native	---	1			---	-------	--	-----
Apo Rat1	1.6	>>	0.095	4.09	8.0	75 ± 24	8.0	48 ± 6
Apo Rat2	1.6	>>	0.077	2.51	8.0	32 ± 5	5.9	65 ± 5
Apo Rabbit	1.6	>>	0.38	8.72	12.2	32 ± 8	9.5	64 ± 20

the binding and activity of the recombined apo-reductase. If we use an apo-sample which does not contain the 78 kDa enzyme (Apo Rabbit) the

association rate constant is only slightly raised. This suggests that the hydrophobic part of the native enzyme does not influence the incorporation velocity of the flavin into the apo-reductase. The K_m values of four other flavins tested are and summarized in Table 3. They are much greater than that of FMN and 6,7 ϕ-FMN. Although the standard deviation is rather great we can see that the affinity for the apo-enzyme is much less than for the FMN and the 6,7 ϕ-FMN.

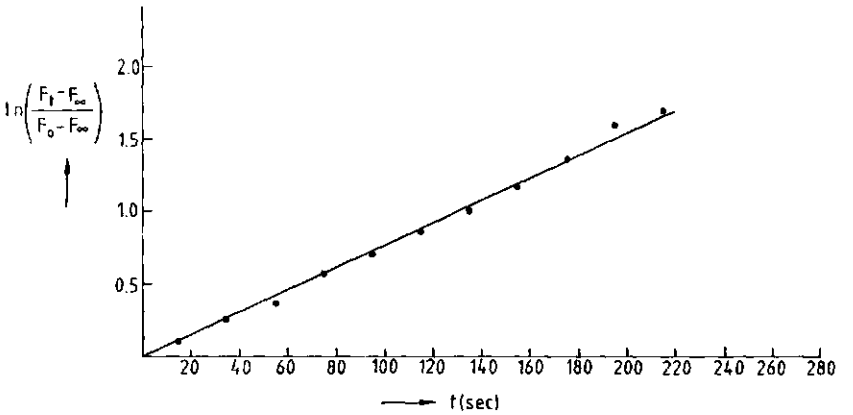


FIGURE 5
Plot of ln $(F_t-F_\infty)/(F_0-F_\infty)$ against time t.
Free FMN in 300 mM potassium phosphate buffer, pH 7.7, was mixed with FMN-depleted reductase in 300 mM potassium phosphate, pH 7.7, and the fluorescence was recorded in time. Temperature was 25°C.
F_0 : fluorescence at time 0;
F_∞ : fluorescence after complete reconstitution;
F_t : fluorescence at time t.
Concentrations : FMN 72.8 nM and FMN-depleted reductase 389 nM.

TABLE 3
Values for K_m and K_m/%Act of different flavins for FMN-depleted NADPH-cytochrome P-450 reductase.

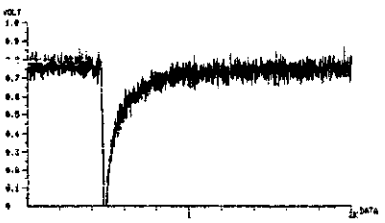
	Apo Rat2		Apo Rabbit	
Flavin	K_m (nM)	K_m/%Act	K_m (nM)	K_m/%Act
FMN	32 ± 5	0.32 ± 0.05	32 ± 8	0.32 ± 0.08
6,7 ϕ-FMN	65 ± 5	0.59 ± 0.05	64 ± 20	0.58 ± 0.18
Riboflavin	180 ± 28	2.5 ± 0.4	82 ± 24	1.14 ± 0.33
3,4-dihydro-FMN	> 300	> 3	> 300	> 3
2-thio-FMN	> 300	> 3	> 300	> 3
3,4-dihydro-6,7-dimethyl-10-ribitylisoalloxazine 5'-phosphate	> 300	> 3	> 300	> 3

The just mentioned method to calculate the second-order association rate constant k_2' is an indirect one. It can be measured directly by measuring the quenching of the fluorescence of the free flavin after

mixing it with the FMN-depleted reductase. Flavin is fluorescent when free in solution and nearly non-fluorescent when associated to the apo-reductase. The apo-reductase (in great excess; in 25 mM potassium phosphate, pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM DTT, 0.1 mM EDTA) is mixed with FMN , so pseudo-first-order kinetics is true. Fig. 5 shows the plot of $\ln (F_t-F_\infty)/(F_0-F_\infty)$ against time t for one concentration of FMN. A pseudo-first-order rate constant k_{ps} of $85 \cdot 10^{-4}$ sec^{-1} has been calculated and a value of $2.2 \cdot 10^4$ $M^{-1}sec^{-1}$ for the association rate constant k_2. This value of k_2 is about four times smaller than the k_2' obtained with the activity measurements. The differences can be explained by the percentage of glycerol present in the measurements. In the absorbance measurements there is no glycerol present and in the fluorescence measurements there is about 6 % glycerol present which accounts for the slower association of flavin with the apo-reductase.

In order to check these values with a different method we repeated both measurements with a stopped flow apparatus. First the kinetics of reconstitution of FMN with the apo-reductase was studied by fluorescence quenching as described in Materials and Methods (D). The decrease of the fluorescence was recorded (see Fig. 6) and after a polynomal smooth-procedure fitted with one exponential decay. The ln of the fluorescence was plotted against the time t (Fig. 7). The slope of this plot gave the

FIGURE 6
Plot of the fluorescence against the time measured by stopped flow. Free FMN in 300 mM potassium phosphate buffer, pH 7.7, was rapidly mixed with FMN-depleted reductase in 300 mM potassium phosphate, pH 7.7, and the fluorescence was recorded in time. Temperature was 25°C.
Final concentrations were: Flavin about 100 nM and FMN-depleted reductase 400-1200 nM.



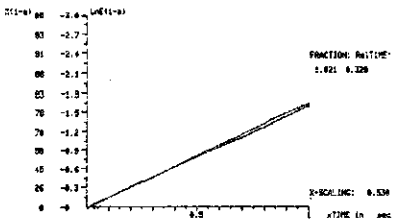


FIGURE 7
Plot of ln(fluorescence) against the time measured by stopped flow. For further explanation see legend Fig. 6.

FIGURE 8
Plot of k_{ps} (pseudo-first-order rate constant) obtained from Fig. 7 against FMN-depleted reductase concentration for FMN.
For further explanation see legend Fig. 6.

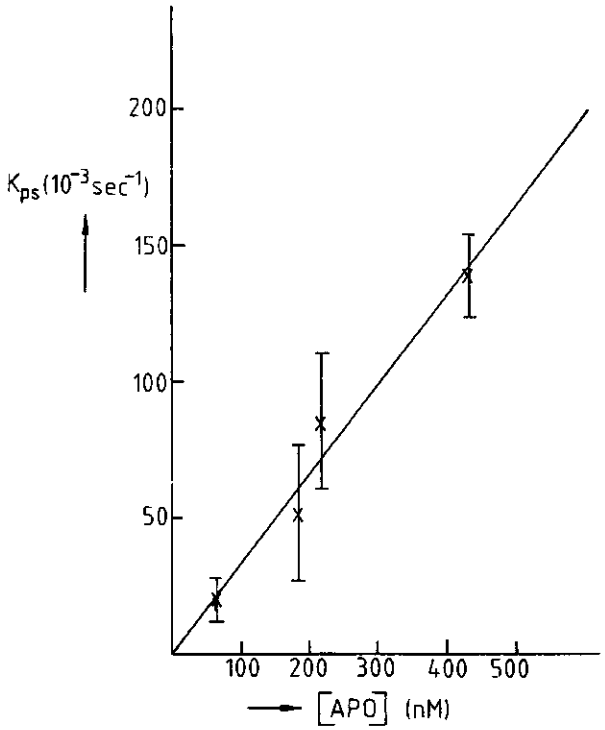


TABLE 4
k_2-values of the kinetics of recombination of different flavins with FMN-depleted reductase as calculated from stopped flow fluorescence measurements.

Flavin	k_2 (10^4 $M^{-1}sec^{-1}$)
FMN	33 ± 3
6,7 ϕ-FMN	20 ± 1
Riboflavin	63 ± 15
3,4-dihydro-6,7-dimethyl-10-ribitylisoalloxazine 5'-phosphate	16 ± 3

pseudo-first-order rate constant kps. This was repeated for several apo-concentrations. Fig. 8 shows a plot of k_{ps} against [apo] for FMN. The association rate constant k_2 could be calculated from the slope of this plot. Table 4 summarizes the k_2 values for different modified flavins. The k_2 for FMN is about 1.5-2 times the k_2 for 6,7 ϕ-FMN. The k_2 for riboflavin is even larger, about 3 times the k_2 for 6,7 ϕ-FMN.

The kinetics of reconstitution of the apo-reductase with modified flavins was also studied by restoring the cytochrome c reduction ability of the enzyme (Materials and Methods (C)) and the dependence of the reaction on the flavin concentration was investigated. The decrease of the transmission at 550 nm (λ_{max} reduced cytochrome c) was monitored (Fig. 9). The plots were smoothed and fitted with one exponential decay. A plot of k_{ps} versus [FMN] (Fig. 10) shows that k_{ps} does not increase anymore at a certain concentration of FMN. The apo-enzyme has been saturated with the flavin and the maximum association velocity has been

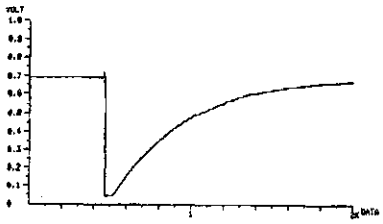


FIGURE 9
Plot of transmission against time as measured by stopped flow.
A solution of cytochrome c,NADPH and flavin in 300 mM potassium phosphate buffer, pH 7.7, was rapidly mixed with FMN-depleted reductase in 300 mM potassium phosphate, pH 7.7, at 25°C. Cytochrome c reduction was recorded at 550 nm. Final concentrations : FMN-depleted reductase 24.5 nM and flavin 200-1000 nM.

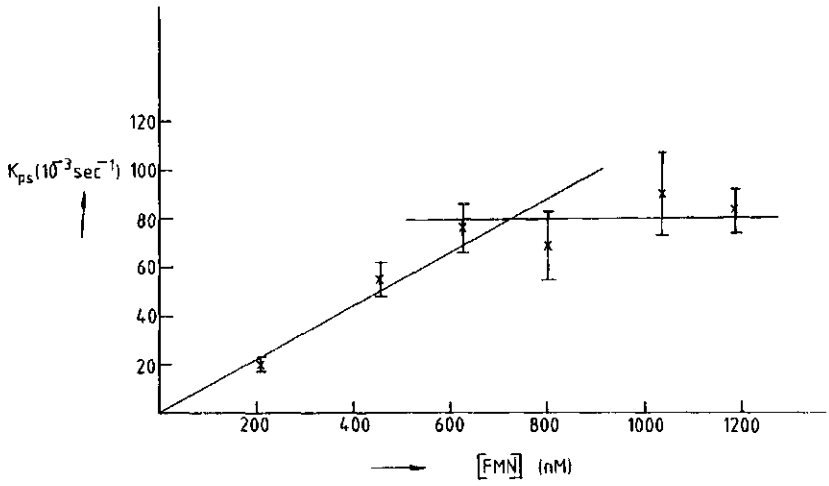


FIGURE 10
Plot of k_{ps} (pseudo-first-order rate constant) obtained from Fig. 9 against FMN concentration. For further explanation see legend Fig. 9.

TABLE 5

k_2'-values of the kinetics of recombination of different flavins with apo-reductase as calculated from stopped flow transmission measurements.

Flavin	k_2' ($10^4 \cdot M^{-1}sec^{-1}$)
FMN	11 ± 2
6,7 ϕ-FMN	6 ± 2
Riboflavin	16 ± 2
3,4-dihydro-6,7-dimethyl-10-ribitylisoalloxazine 5'-phosphate	10 ± 3
7,8 ϕ-FMN	5 ± 2 / 23 ± 5
7,8 ϕ-Riboflavin	15 ± 5

achieved. Table 5 summarizes the k_2' values for the different modified flavins. There is no great difference between the k_2' values of the different flavins. The k_2' values are about the same as obtained by fluorescence measurements.

DISCUSSION

FMN-depleted (apo-) reductase could be reconstituted by different modified flavins to give an active holo enzyme (Table 1).The relative activity of the different flavins shows that 6,7 ϕ-FMN is even more active than native or FMN-reconstituted reductase. An extra phenyl group at the 6,7 position of the isoalloxazine ring is probably of no importance for activity. An extra phosphorus group at the 3' position of the ribityl side chain decreases the activity dramatically to 11% of the activity obtained with FMN. The activity of the apo-reductase reconstituted with the other flavins varied between 70-95%.

The association rate constant k_2' of FMN and 6,7 ϕ-FMN with apo-reductase as measured by cytochrome c reduction of the reconstituted enzyme did not vary much between spectrophotometer and stopped flow experiments. The values for 6,7 ϕ-FMN being slightly lower. The values of k_2' for the other flavins are in the same order of magnitude. The k_2' values for riboflavin and 7,8 ϕ-riboflavin are a little larger. The overal incorporation of the flavins, the binding of cytochrome c and NADPH and the reduction of cytochrome c is therefore faster for the two riboflavins. The plot of k_{ps} against [7,8 ϕ-FMN] (plot not shown) shows clearly two slopes; the two k_2'-values ($5 \cdot 10^4$ and $23 \cdot 10^4$ $M^{-1}sec^{-1}$) suggests the presence of two independent binding flavins.

To measure only the association rate the quenching of the fluorescence

of flavin during the binding to the apo-reductase was studied. The fluorescence of riboflavin is quenched two times faster than that of FMN suggesting that the binding is also two times faster. The affinity on the other hand is about two times lower so it must be concluded that riboflavin dissociates more easily from the enzyme than FMN. Indeed riboflavin dissociates from the enzyme when a riboflavin reconstituted enzyme is subjected to gel chromatography. The association rate constant of FMN is about 1.5-2 times the k_2 of 6,7 ϕ-FMN and is in agreement with the K_m value obtained.

The association rate constant measured by fluorescence is for all flavins about three times greater than measured by the absorption method suggesting that indeed the association of the flavin with the apo-reductase is fast compared with the overal reaction (association of flavin, binding cytochrome c and NADPH, and reduction of cytochrome c). The difference in the k_2-value of FMN for the apo-reductase as measured by conventional fluorescence and by stopped flow techniques ($2.2 \cdot 10^4$ and $33 \cdot 10^4$ M^{-1} sec^{-1}, respectively) can be attributed to the glycerol present in the former.

Vermillion et al. [14] reconstituted apo-reductase with some other modified flavins (7-Br-FMN, 8-Cl-FMN, Iso-FMN, 8-Mercapto-FMN and 5-Deaza-FMN). The activity was more or less regained except for the 5-Deaza-FMN reconstituted enzyme.

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CHAPTER 5

THE PREPARATION AND SOME PROPERTIES OF FMN-DEPLETED NADPH-CYTOCHROME P-450 REDUCTASE

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SUMMARY

In order to investigate the protein-flavin interactions it is necessary to obtain a proper apo-enzyme preparation. A few methods are described for NADPH-cytochrome P-450 reductase. The resulting apo-enzyme and the reconstituted reductase have been studied by different physical techniques.

INTRODUCTION

NADPH-cytochrome P-450 reductase, an enzymatic part of the liver microsomal mixed-function oxidation system, contains two flavins, FMN and FAD, in equimolar quantity [1-6]. It serves as an electron transport enzyme from NADPH to cytochrome P-450. The multi-enzyme system hydroxylates a great deal of different substrates such as steroids, hormones, xenobiotica. After the discovery of the reductase and its function [7-11] there was great interest in the elucidation of the function of the flavins in the reductase. To achieve this goal much attention was paid to the preparation of enzyme containing only one flavin molecule. It turned out that under certain conditions FMN could be removed selectively from the native enzyme, although the dissociation constant for the FMN/FMN-depleted enzyme complex was determined to be 13 nM [12,13]. Since FAD seems not to be removed under the experimental conditions used to prepare FMN-depleted reductase, the affinity of the enzyme for FAD must be much higher than for FMN. Very recently Kurzban and Strobel [14] succeeded in preparing an FAD-free reductase which could be reconstituted to an active enzyme.

The apo-flavoproteins and FMN-depleted NADPH-cytochrome P-450 reductase are generally prepared in small amounts for specific biochemical experiments. For some of our investigations we needed relatively large

amounts of FMN-depleted reductase. This required first to study the optimal conditions under which high quality FMN-depleted protein could be obtained and be reconstituted in high yields. In this paper we describe the evaluation of several procedures for the preparation of FMN-depleted reductase and its physical properties. In addition for the first time in the biochemistry of flavoproteins we present a quantitative description of the preparation of the FMN-depleted reductase.

MATERIALS AND METHODS

Purification:

NADPH-cytochrome P-450 reductase was purified from pig, rabbit and rat liver as described before [1] with some small modifications . SDS gel electrophoresis was performed according to Laemmli [15]. Activity was measured in 300 mM potassium phosphate buffer, pH 7.7, at 25°C by determining cytochrome c reduction at 550 nm ($\Delta\epsilon$= 21 $mM^{-1}cm^{-1}$). Protein determination was carried out by the method of Lowry [16] as modified by Peterson [17].

Procedure 1:

The reductase was bound to either a DTNB-Sepharose column or a Thiopropyl-Sepharose column (equilibrated with 100 mM Tris/ acetate, pH 7.7, containing 10% glycerol, 0.1% lubrol, 1.0 mM EDTA, 0.5 M KCl) by incubation for different times and at different temperatures. Unbound enzyme was removed by washing the column with the equilibration buffer. FMN was removed by washing the column with apo-buffer (see below). After equilibration of the column the FMN-depleted reductase was eluted with 100 mM Tris/acetate, pH 7.7, containing 10% glycerol, 0.1% lubrol, 1.0 mM EDTA, 0.5 M KCl and 20 mM DTT. The eluted FMN-depleted reductase was concentrated and the KBr and DTT (disturbs the activity measurement) were removed by Biogel 6-PDG gel filtration.

Procedure 2:

The reductase was diluted in different apo-buffers (see below). The solution was concentrated with an Amicon apparatus (YM-30 filter) to remove the unbound flavin. After concentration the sample was diluted again with apo-buffer. After repeating this procedure for several times, the concentrated apo-reductase was applied to a Biogel 6-PDG

column (equilibrated with 25 mM potassium phosphate, pH 7.7, containing 10% glycerol, 0.1% lubrol, 0.1 mM DTT, 0.1 mM EDTA) to remove the apo-buffer.

Procedure 3:

The reductase was applied to a Biogel 6-PDG column equilibrated with apo-buffer (see below). Apo-reductase fractions were collected, concentrated in an Amicon apparatus (YM-30 filter) and applied to a Biogel 6-PDG column (equilibrated with 25 mM potassium phosphate, pH 7.7, containing 10% glycerol , 0.1% lubrol, 0.1 mM DTT, 0.1 mM EDTA).

Procedure 4:

This procedure is a combination of procedure 2 and 3 followed by binding of the apo-enzyme to 2'5'ADP-Sepharose. Reductase was diluted in apo-buffer (see below) to 100 ml and concentrated with 2 Amicons (YM-30 filter) of 50 ml ; the concentrate was again diluted to 100 ml and the procedure was repeated. The final concentrate of 5 ml was applied to a 500 ml Biogel column in apo-buffer. Reductase fractions were pooled and concentrated ; KBr was removed by bio gelfiltration (buffer A : 25 mM potassium phosphate, pH 7.7 , containing 10% glycerol, 0.1% lubrol, 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF). The final apo-reductase was divided in four sam-ples : Sample 1 was kept at 4 C (APO 1). Sample 2 was bound to a 1.5 ml 2'5'ADP-Sepharose column, after washing with buffer A , the apo-reductase was eluted with 10 mM 2'& 3' AMP (APO 2) . Sample 3 was bound to a 1.5 ml 2'5'ADP-Sepharose column; the column was washed with 20 volumes of buffer A and the apo-reductase was reconstituted on the column by recirculating buffer A containing FMN during 16 h; after washing the reconstituted reductase was eluted with 2' & 3 AMP (REC 1). Sample 4 was incubated during 16 hours with FMN in buffer A at 4°C, unbound FMN was removed by bio gelfiltration (REC 2).

Apo-buffers used:

Procedure 1:

DTNB-Sepharose : 0.1 M Tris/Acetate, different pH's, containing 0.1% lubrol, 0.1 mM EDTA, 2 M KBr, in the absence or presence of ureum.

Thiopropyl-Sepharose : 0.1 M Tris/Acetate pH 7.7, containing 10 % glycerol, 0.1% lubrol, 0.1 mM EDTA, 2 M KBr,

0.1 mM PMSF.

Thiopropyl-Sepharose : 0.1 M potassium phosphate, pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 2 M KBr , 0.1 mM PMSF.

Procedure 2:

Ultrafiltration : 0.1 M Tris/Acetate pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 2 M KBr, 0.1 mM PMSF.

Ultrafiltration : 0.1 M potassium phosphate, pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 2 M KBr, 0.1 mM PMSF.

Ultrafiltration : 0.1 M Tris/Acetate pH 7.7, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 2 M KBr, 0.1 mM DTT, 0.1 mM PMSF.

Procedure 3:

Biogel 6-PDG : 0.1 M Tris/Acetate pH 7.7, 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 1.0 mM DTT, 2 M KBr, 0.1 mM PMSF.

Procedure 4:

Combined procedure : 0.1 M Tris/Acetaat pH 7.7, containing 20% glycerol, 0.1% lubrol, 2 M KBr, 1.0 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF.

Reconstitution:

FMN-depleted reductase was reconstituted with an excess of FMN at 4°C or 20°C for 15 minutes. The excess of FMN was removed by gel filtration (Biogel 6-PDG).

Circular Dichroism:

Circular dichroism spectra of the holo- and FMN-depleted enzyme were recorded on a Jobin-Yvon Mark V autodichrograph. For the far UV region (190-250 nm) cells of 1mm path length were used ; for the visible region (320-550 nm) cells of 10 mm path length were used.

Analytical ultra centrifugation:

Ultracentrifuge experiments on the holo- and FMN-depleted reductase were performed at 20°C on a MSE Centriscan 75 analytical ultracentrifuge. Runs were analyzed with a home-made minicomputer program

using a HP 85 (Hewlett Packard). Calculations for the molecular weight were done as described previously [18]. Samples were in 100 mM potassium phosphate, pH 7.7, containing 10% glycerol, 0.1% lubrol, 0.1 mM DTT, 1 mM EDTA, 0.1 mM PMSF. Corrections were made for the viscosity.

^{31}P NMR measurements:

^{31}P NMR spectra were recorded with holo-, FMN-depleted- and FMN-reconstituted reductase on a Bruker CXP 300 at 120.8 M Hz and equipped with an Aspec 2000 computer. A quadrature phase detection was employed and field frequency locking was implemented by using deuterium resonance of D_2O (10-20%) in the sample. Spectra were obtained using 10 mm precision NMR tubes (Wilmad 10 mm 7PP). The chemical shifts were determined relative to an external standard of 85% phosphoric acid. Spectra were obtained at 17 ± 2°C in the presence of 0.5W broad-band proton decoupling. The following instrumental settings were used in the acquisition of the free induction decays : 10 µs pulse width, 30 µs delay time, 4800 Hz spectral width, 1.1 s acquisition time, 8 K data points.

Time resolved fluorescence:

Time resolved fluorescence experiments are described elsewhere [19].

Steady state fluorescence:

Steady state fluorescence and anisotropy was measured at 10°C with the photon-counting instrument described by Visser and Santema [20]. Excitation was at 450 nm, emission was measured at 550 nm (cut off filter), 20% density filter. The samples were in 25 mM potassium phosphate, pH 7.7, containing 10% glycerol, 0.1 % lubrol, 0.1 mM DTT, 0.1 mM EDTA. Values are corrected for the buffer.

RESULTS

The purpose of this study was to test several procedures to prepare FMN-depleted NADPH-cytochrome P-450 reductase and to find conditions where an optimal yield of FMN-free reductase could be obtained. Furthermore the FMN stripped protein should also be quantitatively reconstitutable, if possible, to yield a highly active holoprotein. Last, but not

least, the procedure should also be applicable to large amounts of protein.

The procedure described in the literature [12] consists of dialysis of the reductase against a buffer containing 2M KBr at pH 7.7. This is a time consuming procedure which lasts as long as 1 week to obtain the desired modified protein. This long lasting procedure has the disadvantage that the slowly generated FMN-depleted reductase may become structurally altered during this time and yield a low yield of holoprotein. Another disadvantage of the procedure lies in the fact that, when higher concentrations of protein are used, FMN cannot be removed quantitatively in a reasonable time, unless the samples would be dialyzed for several weeks. In the following four approaches for the preparation of FMN-depleted reductase are described and evaluated with respect to yield, stability and reconstitutability of FMN-depleted reductase.

1) Sepharose affinity column:

NADPH-cytochrome P-450 reductase possesses several sulfhydryl groups of which at least one is accessible for chemical modification [21-25]. In a previously published paper we have made use of an

TABLE 1
Conditions to prepare FMN-depleted NADPH-cytochrome P-450 reductase by the DTNB-Sepharose affinity column and the activities of the preparations.

Buffersystem[1)]	pH	[ureum] M	Volume Buffersystem (ml)	% U_-[2)]	% U_+[3)]
Buffer A	7.7		10	34	90
Buffer A	8.0		10	24	39
Buffer A	8.4		10	9	49
Buffer A	8.4		20	8	49
Buffer B	7.7	0.5	10	30	82
Buffer B	7.7	1.0	10	27	68
Buffer B	7.7	1.5	10	17	46
Buffer B	7.7	2.0	10	20	50
Buffer A	7.7		10	34	90
Buffer A	7.7		20	23	72
Buffer A	7.7		100	8	46
Control[4)]	7.7		10	90	92

Columnvolume = 1.5 ml; Binding T = 4°C for 30 minutes ;
Reductase inhomogeneous 78-68-54 kDa, 100 µl on column (1.22 U).
1) Buffer A: 0.1 M Tris/Acetate , containing 0.1% lubrol, 0.1 mM EDTA, 2 M KBr.
Buffer B: Buffer A + ureum
2) % U_- = U_{tot}(-fmn)/ 1.22 * 100;
U_{tot} (-fmn): total activity FMN-depleted reductase without FMN.
3) % U_+ = U_{tot}(+fmn)/ 1.22 * 100;
U_{tot} (+fmn): total activity FMN-depleted reductase with FMN.
4) Holoreductase was bound; the column was washed with equilibration-buffer instead of apo-buffer and the reductase was eluted .

accessible SH-group to prepare high quality apo-p-hydroxybenzoate hydroxylase by covalent binding of the protein to a DTNB-Sepharose column [26]. The same procedure was now applied to the reductase. 1.22 units were brought on the column. After incubation for 30 minutes the unbound reductase was removed from the column by washing with 7 bed volumes of the incubation buffer, releasing about 0.3 unit of enzyme. FMN was then stripped of the covalently bound enzyme by the use of various buffer systems (Table 1). From Table 1 it is evident that ureum does not have a large influence in the yield of formation of FMN-depleted enzyme. On the other hand increasing the pH value from 7.7 to 8.4 yields a preparation containing about 10% of the original FMN content. A similar effect could be achieved by the use of a larger volume of incubation buffer. The reconstitutability of various preparations is also shown in Table 1. The results show that the modified enzyme reconstitutes to a lesser degree if most of the FMN is released from the protein. That this is not due to instabilization of the enzyme when bound to the affinity column is clearly demonstrated by the results obtained from control experiments (Table 1). It should also be mentioned that the binding capacity of the affinity column decreased after several uses.

TABLE 2
The preparation of FMN-depleted NADPH-cytochrome P-450 reductase under various conditions using Thiopropyl-Sepharose as an affinity column and the quantitation of the prosthetic groups and the activity of the FMN-depleted reductase in the various preparations.

Sample	U[1] -fmn (U)	U[1] +fmn (U)	pro-tein (mg)	FAD (nmole)	FMN (nmole)	a[2] -fmn (U/mg)	a[2] +fmn (U/mg)	%FAD[3]	%FMN[4]	pro-tein %
Native	105	122	1.6	22.2	14.8	65.6	76.3	108	72	100
Apo Tp1	4	10	0.7	2.2	1.6	5.5	14.4	26	18	42
Apo Tp2	2	11	1.0	2.9	0.5	1.9	11.3	23	4	63
Apo Tp3	0	1	1.3	0.6	1.4	0	1.1	4	9	78
Apo Tp4	0.5	2	0.3	0.8	0.2	1.5	5.3	18	3	21

Column volume = 10 ml; binding T = 20°C for 2 hours .
Reductase homogeneous 78 kDa, 1 ml on column (122 U) .
Buffer (for Tp1 and Tp2) : 0.1 M Tris/Acetate pH 7.7, 0.1% lubrol, 10% glycerol, 0.1 mM EDTA, 2 M KBr, 0.1 mM PMSF
Buffer (for Tp3 and Tp4) : 0.1 M potassium phosphate, pH 7.7, 0.1% lubrol, 20% glycerol, 0.1 mM EDTA, 2 M KBr, 0.1 mM PMSF
Volume apo-buffer : 100 ml.
1) U(-fmn), U(+fmn) : activity (apo)reductase in the absence and presence ,respectively, of an excess of FMN.
2) a(-fmn), a(+fmn) : activity (apo)reductase in the absence and presence , respectively, of an excess of FMN/mg protein.
3) %FAD = FAD(nmole)/ protein (nmole) * 100.
4) %FMN = FMN(nmole)/ protein (nmole) * 100.

The related affinity column material Thiopropyl-Sepharose was also tested. It turned out that the enzyme behaved similar as on DTNB-Sepharose. The Thiopropyl-Sepharose column was used for further studies to investigate the properties of the bound enzyme. In this experiment glycerol was added to the buffer systems to stabilize the membrane-bound enzyme. The results are summarized in Table 2. It can be seen that besides FMN also FAD has been removed to a considerable degree. In the literature [23-25] it is mentioned that a relative easy accessible SH group is important for the FAD-protein interaction. If this SH group reacts with the column material or with the apo-buffer it weakens the SH-FAD interaction and FAD could dissociate from the protein. This is a reasonable explanation of our results. Furthermore the circular dichroism spectrum in the far UV-region of the enzyme in the buffer (100 mM Tris/HCl, pH 7.7, containing 10% glycerol, 1 mM EDTA, 0.1 mM DTT , 2 M KBr) indicates that the conformation of the enzyme differs from that of the native enzyme in the same buffer, but in the absence of KBr (spectra not shown). These results suggest that especially the presence of KBr in the buffer systems also influence the FAD-protein interaction, a not unexpected result. Evident is also that some protein has been lost, but the loss of FAD is substantial greater. However, an even larger disadvantage of the above procedure is that the degree of reproducibility varied much and therefore these procedures are not useful for the preparation of FMN-depleted reductase.

2) Ultrafiltration with an Amicon apparatus:

Since the enzyme-FMN interaction has a finite equilibrium it should be possible to remove FMN from the enzyme by dilution experiments. Therefore a concentrated solution of native enzyme was diluted with buffer systems of different composition and concentrated by ultrafiltration. This procedure was repeated several times. Experiences learned that a five to six times repetition of the procedure yielded a preparation containing only a small amount of FMN. This sample was then divided into equal volumes. One sample was analyzed immediately, the other was incubated for further 72 h. As shown in Table 3 both samples lost also FAD, although to a different degree. There is no loss of protein. If we express in these experiments the specific activity as units/nmol FAD instead of units/mg protein, then the specific activity of Apo Am1 and of Apo Am3 after reconstitution with FMN (1.86 and 1.93 U/nmole, respectively) is much less than

TABLE 3
The preparation of FMN-depleted NADPH-cytochrome P-450 reductase under various conditions using the ultrafiltration procedure and the quantitation of the prosthetic groups and the activity of the FMN-depleted reductase in the various preparations.

Sample	U[1] -fmn (U)	U[1] +fmn (U)	protein (mg)	FAD (nmole)	FMN (nmole)	a[2] -fmn (U/mg)	a[2] +fmn (U/mg)	%FAD[3]	%FMN[4]	%protein
Holo	105	122	1.7	22.2	14.8	61.0	70.9	101	67	100
Apo Am1*)	6	31	1.3	16.8	1.7	4.3	24.4	102	10	74
Apo Am2+) 105	3	13	1.0	6.6	0.6	2.8	13.6	55	5.0	
Apo Am3+)	0	± 0	0.9	0.4	<0.1	0	<0.1	3	0.5	

*) 50 ml Amicon : concentration from 50 to 5 ml (6 times)
Reductase homogeneous 78 kDa (1 ml; 122 U)
Buffer : 0.1 M Tris/Acetate pH 7.7, 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 2 M KBr, 0.1 mM PMSF.

+) 50 ml Amicon : concentration from 50 to 5 ml (5 times); for further procedure see text. Reductase homogeneous 78 kDa (1 ml; 122 U)
Buffer : 0.1 M potassium phosphate, pH 7.7, 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 2 M KBr, 0.1 mM PMSF.

1) U(-fmn), U(+fmn) : activity (apo)reductase in the absence and presence , respectively, of an excess of FMN.

2) a(-fmn), a(+fmn) : activity (apo)reductase in the absence and presence , respectively, of an excess of FMN/mg protein.

3) %FAD = FAD(nmole)/ protein (nmole) * 100.

4) %FMN = FMN(nmole)/ protein (nmole) * 100.

TABLE 4
The preparation of FMN-depleted NADPH-cytochrome P-450 reductase under various conditions using the ultrafiltration procedure and the quantitation of the prosthetic groups and the activity of the FMN-depleted reductase in the various preparations.

Sample	U[1] -fmn (U)	U[1] +fmn (U)	FAD (nmole)	FMN (nmole)	U(+fmn)/FAD[2] (U/nmole)	FAD/FMN	%FAD[3]
Holo	78.4	95.2	49.7	36.6	1.9	1.4	100
Apo Am4 *)	7.5	11.0	23.6	9.5	0.5	2.5	47
Apo Am5 *)	5.3	20.0	17.0	0	1.2	>10	34

*) 50 ml Amicon : concentration from 50 to 5 ml (5 times),for further procedure see text. Reductase homogeneous 78 kDa (1 ml; 95.2 U)
Buffer : 0.1 M Tris/Acetate pH 7.7, 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 2 M KBr, 0.1 mM DTT, 0.1 mM PMSF.

1) U(-fmn), U(+fmn) : activity (apo)reductase in the absence and presence , respectively, of an excess of FMN.

2) U (+fmn)/FAD : activity (apo)reductase in the presence of an excess of FMN/ nmole FAD.

3) % FAD : (nmole FAD(apo) / nmole FAD(holo)) * 100%.

that of the native reductase (5.50 U/nmole). As already mentioned above a SH group is involved in the binding of FAD. It is possible that this SH group becomes oxidized during the above described experiments and is the cause for the easy release of FAD from the enzyme. To check this possibility experiments were done where to the buffer systems DTT was added to prevent oxidation of the SH group. The results are summarized in Table 4. Again the initial sample of FMN-depleted reductase was divided into two

equal volumes. One sample was measured immediately (Apo Am4) and the other was measured after 16 hours incubation in apo-buffer + 1mM DTT (Apo Am5). From Table 4 we can conclude that increasing the DTT-concentration in the apo-buffer leads to an improvement of the specific activity of the apo-reductase after FMN-addition, expressed as U/nmol FAD. Even after two days of incubation there was no loss of FAD. These results strongly suggest that the SH group, which is important for FAD binding, has been protected during incubation .

3) Gel chromatography:

Procedure 3 was used to make reductase FMN-depleted in 4 experiments with 2 Biogel columns. We varied the incubation time by means of different flow rates. The specifications of the columns are given in Table 5. Table 6 gives a resumee of the results. The small columns

TABLE 5
Specifications Biogel columns apo-procedure Biogel.

Procedure	Volume column (ml)	Flow (ml/h)	Height (cm)	O[1] (cm^2)	Flow (ml/cm^2h)	Volume sample (ml)	t incub. (hour)
BIO 1	80	12	14	5.7	2.1	2.5	5
BIO 2	80	29	14	5.7	0.51	2.0	11
BIO 3	500	80	94	5.3	15	1.6	5
BIO 4	500	100	94	5.3	19	1.9	5

Apo-buffer : 0.1 M Tris/Acetate pH 7.7, 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, 1.0 mM DTT, 2 M KBr, 0.1 mM PMSF.
Reductase : inhomogeneous 78,70,50 kDa (BIO 1, BIO 2).
homogeneous 78 kDa (BIO 3, BIO 4).
1) O : surface area of column.

give only a removal of 50% of FMN, although the specific activity of the apo-samples (U_+/nmolFAD) is the same as that of the holo-sample even after 11 hours incubation time. Remarkable with the large columns is that for an incubation time of 5 hours, in both experiments, the specific activity of the apo-samples is substantially lower than in the holo-reductase. The FMN removal was also not complete but better than with the short columns. The apo-sample of experiment 4 (Apo BIO4) was again loaded on the large column and eluted with the same apo-buffer. The resulting apo-reductase (Apo BIO5) was almost FMN-free. The specific activity after recombination was on the contrary further decreased. To prove if the decrease of the specific activity was due to incomplete

TABLE 6
Results apo-procedure Biogel columns.

Sample	U[1] -fmn (U)	U[2] +fmn (U)	conc[3] red (μM)	FAD (nmole)	FMN	U(+fmn)/FAD (U/nmole)	FAD/FMN	%FAD[4]
Holo 1	82.3	159	21.0	52.6	24.2	3.02	2.11	100
Apo BIO1	41.0	121	7.04	42.2	12.1	2.87	3.48	80
Holo 2	165	234	40.2	80.4	58.6	2.91	1.37	100
Apo BIO2	84.8	172	8.72	69.8	28.4	2.46	2.46	87
Holo 3	302	382	57.2	92.0	77.9	4.15	1.18	100
Apo BIO3	77.4	145	13.4	80.4	18.0	4.46	1.80	87
Holo 4	135	165	26.5	50.4	40.7	3.29	1.24	100
Apo BIO4	29.6	69	7.44	37.2	9.28	1.85	4.01	74
Apo BIO4	9.26	24.2	7.44	13.0	3.26	1.85	4.11	100
Apo BIO5	2.54	9.76	4.77	9.54	0.44	1.02	>10	73

For specifications see Table 7.
1) U(-fmn) : total activity (apo)reductase without FMN.
2) U(+fmn) : total activity (apo)reductase with FMN was measured after incubation for 10' at 25°C with an excess of FMN.
3) Conc red : calculated as concentration FAD.
4) % FAD : (nmole FAD(apo) / nmole FAD(holo)) * 100%.

TABLE 7
Results of recombination of samples apo-procedure biogel columns.

Sample	U(+fmn)/FAD[1] (U/nmole)	U(-fmn)/FMN[2] (U/nmole)	FAD/FMN
Holo BIO 1	3.02	3.40	2.11
Rec BIO 1	3.40	3.53	1.12
Holo BIO 2	2.91	2.82	1.37
Rec BIO 2	2.58	2.51	1.04
Holo BIO 3	4.15	3.88	1.18
Rec BIO 3	2.18	2.68	1.58
Holo BIO 4	3.29	3.31	1.24
Rec BIO 4	2.06	2.62	1.37

1) U(+fmn)/FAD : total activity (apo)(rec)reductase after incubation with an excess FMN / nmole FAD.
2) U(-fmn)/FMN : total activity (apo)(rec)reductase without FMN / nmole FMN.

recombination the samples were incubated with FMN during 16 h. After removal of unbound FMN the samples were measured (Table 7); the FAD/FMN ratio were for all recombined samples ± 1 and the specific activities returned for the small biogel columns to almost 100% and for the large columns to 60-70% of the initial holo specific activity. The reason for the bad reconstitution of the apo-reductase obtained by the large biogel column can be twofold : The reductase used in the small biogel procedure was impure and contained the 76.000 Da, 69.000 Da and the 50.000 Da part of the enzyme. In the other procedure the reductase was pure 76.000 Da. Perhaps the 76.000 Da enzyme recombines less efficiently than the inhomogeneous sample. Another possible reason lies in the concentration of lubrol present in the apo-sample; after the biogel column the reductase

fractions are pooled and concentrated. In case of the small column the sample is less in volume than in the case of the large column. If lubrol is beyond its critical micel concentration it doesn't pass the YM-30 filter and concentration of lubrol is raised. The larger the volume of the apo-sample the higher the concentration of lubrol will become. The apo BIO 4 sample has therefore a higher lubrol concentration and this might be the reason for the less reconstitution of this sample. Therefore we applied the apo-sample to the 2'5'ADP Sepharose column to remove the lubrol.

4) Amicon, Gelchromatography and 2'5' ADP Sepharose:

Procedure 4 (Materials and Methods) was followed to make reductase FMN-depleted and reconstitute it with FMN. The results of this experiment are summarized in Table 8 and Table 9. The recovery of protein (51%)

TABLE 8
Results of the combined apo-procedure.

Sample	U[1] -fmn (U)	U[1] +fmn (U)	pro-tein (mg)	FAD (nmole)	FMN	a[2] +fmn (U/mg)	U_+/FAD[3] (U/nmole)	%FAD[4]	%FMN[5]	%pro-tein
Holo	126	156	6.06	61.2	46.4	25.7	2.56	79	60	100
APO 1	6.1	49.9	3.11	37.2	0	16.0	1.34	93	0	51
APO 2	16	70.9	2.64	30.8	3.7	29.8	2.56	91	10	--

Apo-buffer : 0.1 M Tris/Acetaat pH 7.7, 20% glycerol, 0.1% lubrol, 1.0 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF.
1) U(-fmn), U(+fmn) : activity (apo)reductase in the absence and presence , respectively, of an excess of FMN.
2) a(+fmn) : activity (apo)reductase in the presence of an excess of FMN/ mg protein.
3) U_+/FAD : total activity (apo)(rec)reductase after incubation with an excess FMN / nmole FAD.
4) %FAD = FAD(nmole)/ protein (nmole) * 100.
5) %FMN = FMN(nmole)/ protein (nmole) * 100.
Values corrected for partition.

TABLE 9
Results of recombination of samples combined apo-procedure.

Sample	U(+fmn)/FAD[1] (U/nmole)	U(-fmn)/FMN[2] (U/nmole)	a(+fmn)[3] (U/mg)	FAD/FMN	%recovery[4]
Holo	2.56	2.72	25.7	1.31	--
Rec 1	0.91	2.71	13.5	3.46	78
Rec 2	1.38	3.61	14.3	3.10	--
APO 2	2.56	4.45	29.8	8.78	85

1) U(+fmn)/FAD : total activity (apo)(rec)reductase after incubation with an excess FMN / nmole FAD.
2) U(-fmn)/FMN : total activity (apo)(rec)reductase without FMN / nmole FMN.
3) a(+fmn) : activity (apo)reductase in the presence of an excess of FMN/ mg protein.
4) % recovery : (mg protein bound to ADP-column/ mg protein eluted from 2'5'ADP-Sepharose column) * 100%.

was rather low ; the reason for this is unknown . Remarkable is the specific activity of the apo-sample bound to 2'5'ADP Sepharose (APO 2) which is the same as the one of the holo-reductase . Reconstitution is 100%, which can't be said of the other samples. The reconstitution of APO 1 succeeded for no more than 52%. Probably the conformation for reconstitution after the 2'5'ADP Sepharose column is ideal or the removal of lubrol is the essential point for 100% reconstitution.

5) A model for the procedure by the Amicon:

A model is described by which we can calculate the percentage of FMN-depleted reductase when we follow Procedure 2. The system is discrete. During incubation of the reductase with KBr an equilibrium between bound and free FMN is established. During concentration the free FMN is removed from the system; the concentration of free FMN remains constant, also the ratio [FMN-free]/ [holoreductase]. After refilling of the Amicon a new equilibrium establishes. In Figure 1 the equation is derived by which we can calculate the percentage of FMN-depleted reductase. If the start and end situation of the apo-procedure is known we can

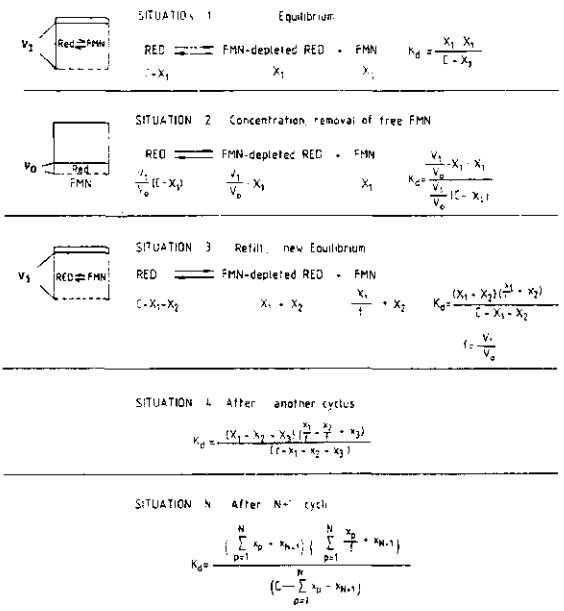


FIGURE 1
Scheme of the procedure to make reductase FMN-depleted by ultrafiltration and calculation of the percentage of FMN-depleted reductase after several steps.

FIGURE 2
Simulation of the K_d of the FMN / protein interaction and calculation of the percentage of FMN-depleted reductase (R) after several steps (n) of the procedure to make FMN-depleted reductase by ultrafiltration.
Reductase concentrations are: A) $4.44 \cdot 10^{-7}$ M; B) $4.96 \cdot 10^{-6}$ M.

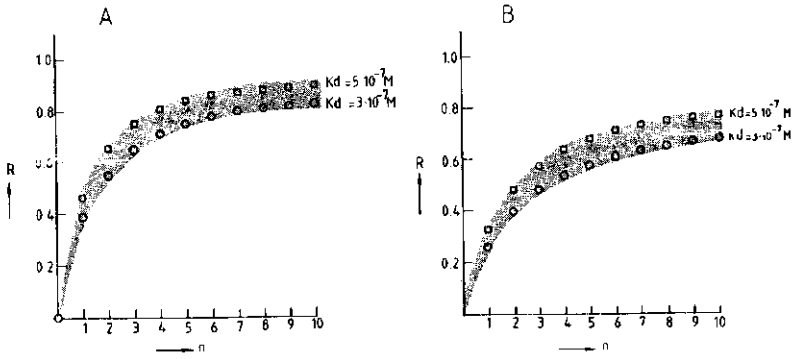
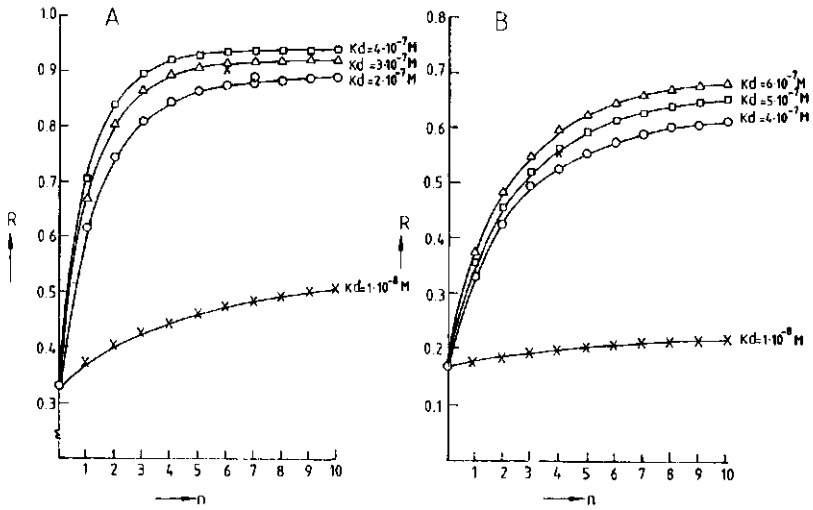


FIGURE 3
Calculation of the percentage of FMN-depleted reductase (R) after several steps (n) of the procedure to make FMN-depleted reductase by ultrafiltration for two amounts of enzyme: A) 40 mg; B) 100 mg.



calculate the K_d in 2 M KBr. Two experimental situations (number of cycli and reductase concentration are known) are fitted with an estimated K_d. The FMN and FAD content of the apo-sample are measured so that we can calculate the percentage of FMN-depleted reductase. Figure 2 represents a number of simulations in which the K_d has been varied for 2 Amicon experiments. The K_d's for both experiments agree with each other. If we

take these values as boundaries for the K_d than we can calculate the experimental parameters for an apo-procedure for any amount of reductase in this apo-buffer. In Figure 3 two of such simulations are represented. We see that 100 mg of reductase in a 400 ml Amicon with 10 cycli and f=20 (400 ml concentrated to 20 ml) becomes FMN-depleted for 68-77%. The curve becomes flat and more cycli doesn't improve the result, so it is better to make FMN-depleted reductase in small amounts.

6) Characterization of the Apo- and the FMN-recombined reductase:

6.1) Circular Dichroism.

In the visible range of the CD-spectrum the holo-reductase shows two bands with negative Cotton effect, one at 445 nm and one at about 350 nm (Figure 4). Apo-reductase on the contrary shows a band with positive dichroism at 370-380 nm; the band at 350 nm has been diminished and the band at 445 has about the same rotation strength as in the holo-reductase. Both the FAD and the FMN show therefore strong induced dichroism on association with the reductase. From the difference of the CD-spectra of holo- and apo-reductase in the visible range we can conclude that both chromofores are located in a different environment. The strong indu-

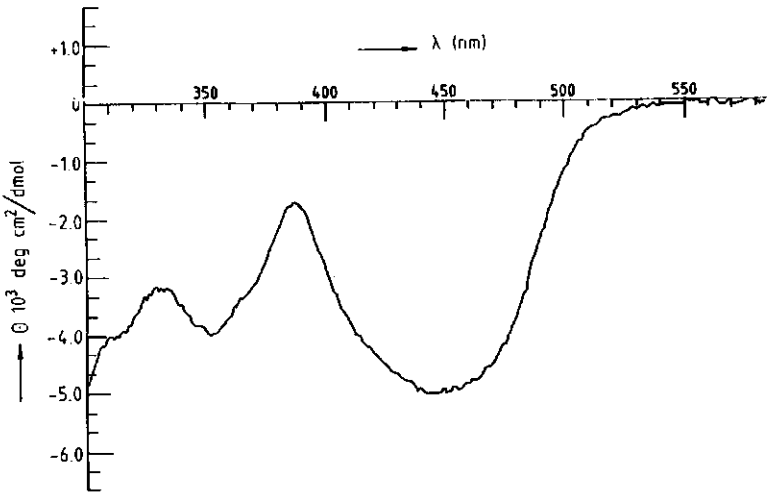


FIGURE 4
CD spectrum of the native NADPH-cytochrome P-450 reductase in the visible range (320-550 nm).

ced dichroism arises from a asymmetric milieu on both sides of the isoalloxazine ring. The negative band at 350 nm can be attributed to FMN

if the position of FAD in the protein did not change on removal of FMN. On recombination of the apo-reductase with FMN the 350 nm band is less pro-nounced , while the 445 nm band is increased clearly. The question arises if the FMN is located in the same way as in the holo enzyme. Other papers will be dealing more in detail on this issue [20,27].

In the far UV region of the CD-spectrum (Figure 5) we can calculate with the help of the reference spectra of Fasman [28], Wetlaufer [29] and Chen [30] the percentage of α-helix, β-sheet and random coil of the different reductase-samples ; these are summarized in Table 10 and we

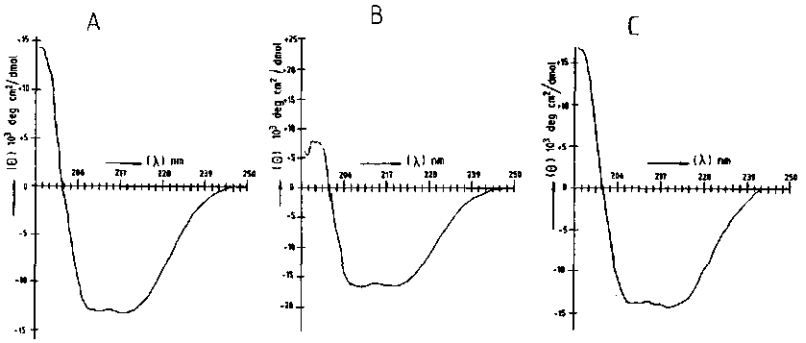


Figure 5
CD spectrum of NADPH-cytochrome P-450 reductase in the far UV-region (190-250 nm). A) native; B) FMN-depleted; C) FMN-reconstituted.

TABLE 10
Results of the Circular Dichroism measurements.
Percentages of conformation of different samples of NADPH-cytochrome P-450 reductase as calculated by Fasman [28], Wetlaufer [29] and Chen [30].

Sample		Conformation	Fasman %	Wetlaufer %	Chen %
Holo	4°C	α-helix	16	20	37
		β-sheet	53	38	23
		random coil	34	42	40
Holo	12°C	α-helix	14	20	36
		β-sheet	56	41	27
		random coil	30	39	37
Holo	23°C	α-helix	14	20	36
		β-sheet	56	41	27
		random coil	30	39	37
Holo	30°C	α-helix	8	20	34
		β-sheet	59	38	25
		random coil	33	42	41
FMN-depleted[1]		α-helix	35	28	46
	20°C	β-sheet	45	46	24
		random coil	20	25	30
REC-FMN[2]		α-helix	27	27	41
	20°C	β-sheet	42	39	20
		random coil	30	33	35

1) FMN-depleted reductase
2) FMN-reconstituted FMN-depleted reductase

see that the values for the different methods are divergent. The set of Fasman gave for lumazine protein very inconsistent values [31], the

set of Chen has the property of pronouncing the α-helix percentage with regard to the set of Wetlaufer. In any case the values of the different samples are comparible if one uses the same set of reference spectra. The apo-reductase gives for the three sets a more structured protein than the holo-protein. The FMN recombined reductase gives a structure which is between apo and holo. It seems that the conformational change, although it is not much, is reversible. In the literature a value of 29% is given for the percentage of α-helix [32]. Our value of 29-30% is in good agreement with this.

6.2) ^{31}P NMR.

^{31}P NMR spectra of native reductase, FMN-depleted reductase and FMN-recombined reductase are given in chapter 2 [27]. These spectra show that the FAD phosphate resonances have been changed during the apo-procedure ; they become sharper, which suggests a weaker interaction of those phosphates with the protein. The FAD-resonances return slowly to the native state after recombination with FMN.

6.3) Ultracentrifuge.

Ultracentrifuge experiments were performed to see if the reductase in the different configurations forms aggregates. Aggregation of both the holo-reductase and the apo-reductase has a disturbing influence on activity , removal of FMN, reconstitution of FMN with the apo-enzyme, etc.

We looked at 3 different aspects :

- sedimentation behaviour of homogeneous native reductase (78 kDa) vs that of inhomogeneous (78,70,50 kDa) reductase.
- the effect of ionic strength on the aggregration of 78 kDa reductase.
- the influence of FMN-removal on the sedimentation behaviour of native reductase.

Figure 6A shows the sedimentation pattern of homogeneous reductase of 78 kDa. One singel sedimenting fragment can be seen indeed . The sedimentation coefficient agrees with the molecular weight as determined with PAGE (poly acrylamide gel electrophoresis). The sedimentation pattern of inhomogeneous reductase (Figure 6B) shows also the pattern as predicted by PAGE. The sedimentation patterns of homogeneous reductase in different concentrations of potassium phosphate buffer shows no aggregation at all (data not shown). Also by lowering the temperature no aggregation can be

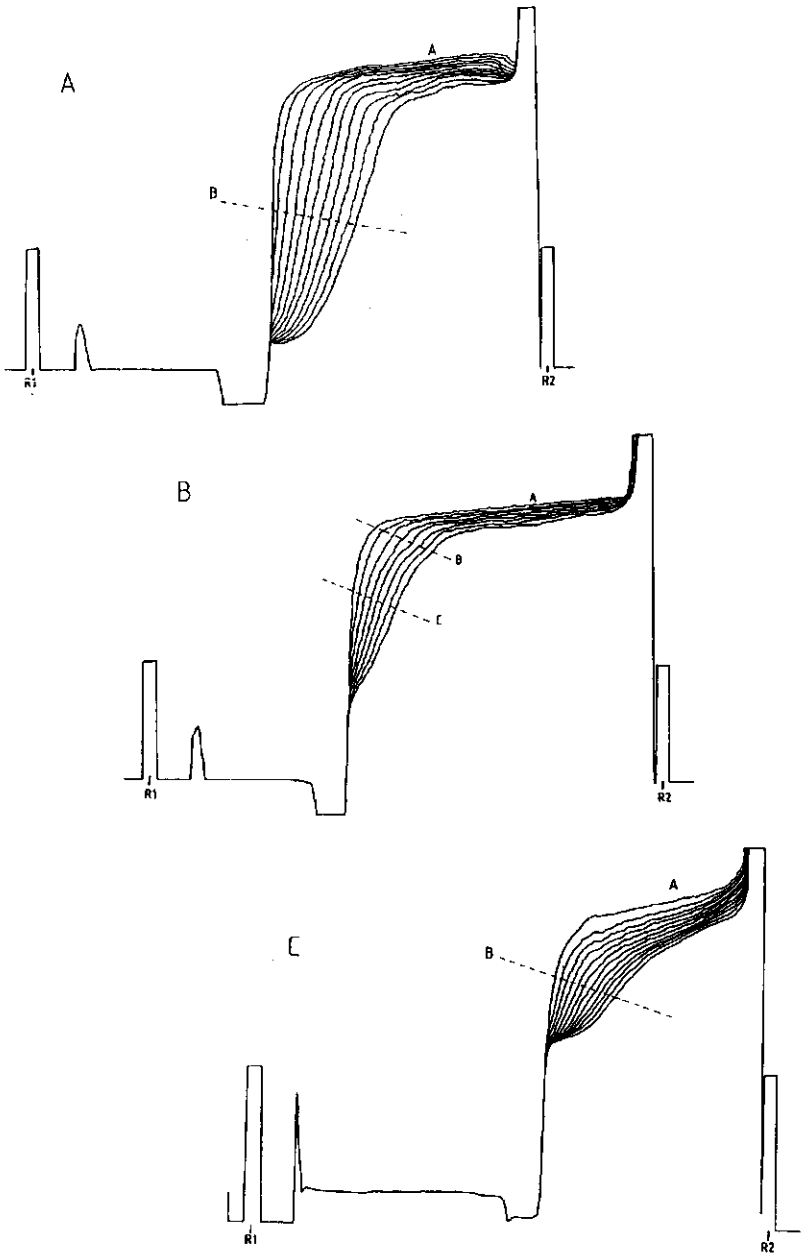


Figure 6
Ultra centrifugation patterns of different NADPH-cytochrome P-450 reductase preparations. (A) Native reductase 78 kDa; (B) Inhomogeneous reductase; (C) FMN-depleted reductase after being frozen at -70°C. A,B,C in the different figures represent different sedimenting species.

seen (data not shown). The FMN-depleted reductase (homogeneous 78 kDa) shows a homogeneous sedimentation pattern with a sedimentation coefficient slightly lower than that from the native enzyme (data not shown). Figure 6C shows the sedimentation pattern of the apo-reductase after being frozen at -70°C. A substantial part of the apo-enzyme has been aggregated or denatured. The calculated sedimentation coefficient of the holo-reductase corresponds almost with that of BSA (68 kDa) which means that holo- and FMN-depleted reductase exist in vitro as monomers.

6.4) Time resolved fluorescence spectroscopy.

In chapter 3 this item has been discussed [19].

6.5) Steady state fluorescence.

Steady state fluorescence was measured on 3 different samples : holo-, apo- and FMN-recombined reductase. Also the steady state anisotropy was measured at the same time. Table 11 shows the polarization and the anisotropy of the different samples. The results show that the holo- and the apo-reductase both have a high steady state anisotropy. The recombined sample has a quite low steady state anisotropy (0.130) and it becomes even lower as time increases. The activity on the other hand remains the same. Our conclusion is that FMN is not reconstituted in the proper way otherwise the steady state polarization and -anisotropy of the recombined reductase should be the same as that of the holo. The FMN of the recombined sample becomes looser in time although it has no effect on the activity.

TABLE 11
Results measurements of steady state anisotropy.

Sample	Time[1] (hours)	Polarization	Anisotropy	A[2] (-fmn) (U/ml)	A[2] (+fmn) (U/ml)
Holo	--	0.435	0.339	108.4	155.6
Holo	24	0.468	0.370	127.1	194.1
Apo	--	0.441	0.345	21.6	50.6
Apo	48	0.433	0.337	9.96	59.1
Rec 1	1	0.184	0.130	41.9	46.2
Rec 2	4	0.138	0.097	35.8	43.3
Rec 3	24	0.113	0.078	41.5	49.8

1) Time after measurement of polarization,anisotropy,activity.
2) A(-fmn),A(+fmn) : activity (apo)(rec)reductase without resp. with an excess of FMN.

DISCUSSION

NADPH Cytochrome P-450 reductase, a flavoprotein which contains FAD and FMN, transports electrons from NADPH to cytochrome P-450. Both flavins play an essential part in this process. FAD is the entry part of the reductase for the electrons and FMN the exit part. In this paper we removed the FMN in several ways and studied the apo- and the reconstituted reductase by different techniques. The different procedures for preparing the apo enzyme gave some remarkable results : The procedure to make the reductase FMN-depleted by binding on a DTNB- or a Thiopropyl column has not been successful because FAD dissociates from the enzyme. High concentrations of DTT prevents this dissociation by keeping a certain SH-group reduced which interacts probably with the FAD. This was shown in the Amicon apo-procedure. For this procedure an equation was derived to calculate the percentage of FMN-depleted reductase for a certain concentration of reductase in the Amicon and a certain number of cycles. It was shown that for large amounts of reductase it is quite impossible to get an almost FMN free apo-preparation of the reductase. This also applies for the apo-procedure by gelfiltration or the dialysis method as described by Vermillion and Coon [12].

The degree of reconstitution ability of the apo-enzyme for an active enzyme again, varies between the different procedures. In any case it is clear that a high concentration of DTT (1 mM) is essential to get an apo-enzyme which could be reconstituted to an active enzyme.

The combined procedure (Amicon,Gel filtration,2'5'ADP Sepharose) shows a remarkable good apo sample after binding on the 2'5' ADP Sepharose column. The specific activity (U_+/nmole FAD) after reconstitution is 100%. This all makes this procedure the most favourable one. The gel filtration step could be omitted in this procedure . The characterization of the holo- , apo- and FMN-recombined reductase has been performed with different physical techniques. The Circular Dichroism spectra in the visible region show that the FMN in the recombined reductase is not in the same environment as in the holo enzyme. This result was also obtained by time resolved- and steady state fluorescence measurements. The fact that activity on the other hand is still nearly 100% is strange. This means that FMN does not need to be bound in a certain way to measure activity. The CD spectra in the far UV region of the holoreductase shows a certain degree of conformational change of the protein in the KBr

buffer, which is reversible on removal of the KBr. The apo-reductase shows a slightly different conformation as deduced from its CD spectrum.

Ultracentrifuge experiments show no aggregation of holo- and apo-reductase. Only if the apo-enzyme has been frozen at -70°C , the sedimentation pattern shows some aggregation.

The FAD environment becomes altered by the apo-procedure as shown by ^{31}P NMR studies.

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SUMMARY

The research on flavins and flavoproteins started in 1879 with the discovery of the yellow pigment "lactochrome" in whey by Blyth. Later it turned out that "lactochrome" and other isolated yellow pigments were derivatives of riboflavin (vitamin B_2). The first as such recognized flavoprotein was the "old yellow enzyme" NADPH dehydrogenase, which was isolated from yeast in 1932. At the moment about hundred flavoproteins are known, which contain FAD and/or FMN as prosthetic group. The flavin coenzyme can be covalently or noncovalently bound to the enzyme. Their function is different . Some flavoproteins act as dehydrogenases, while others are oxygenases. Müller wrote in 1981 a review on the different flavoproteins present in nature.

The enzyme discussed in this thesis, NADPH-cytochrome P-450 reductase, contains two flavins : FAD and FMN, which are both noncovalently bound to the protein.

Chapter 1 contains a broad review on this particular flavoprotein and the reader is referred to this for further information. A short survey of the enzyme will be on its place here. NADPH-cytochrome P-450 reductase, containing both FAD and FMN as prosthetic groups, was first discovered by Horecker in 1949, although he was not aware of it. Later other investigators were involved in isolating the enzyme to homogeneity and proving its function. The NADPH-cytochrome P-450 reductase is a membrane bound flavoprotein, which is particularly present in the endoplasmatic reticulum of the liver. The enzyme is part of the mixed function oxidase system and transports electrons from NADPH to cytochrome P-450 by means of its flavins . The multi-enzyme system cytochrome P-450 plays an important role in the detoxification of xenobiotica, activation of procarcinogens, the steroid- and fatty acid metabolism etc. The essential reaction is the hydroxylation by cytochrome P-450. The amino acid sequence of the 78 kDa protein has been determined. It turned out that there was some degree of homology between the reductase and some other flavoproteins . The redox potentials of the individual flavins have been calculated. FMN and FAD could be removed from the enzyme and it was also possible to restore activity after reconstitution of the flavin-depleted enzyme with FMN or FAD. A 68 kDa proteolytic fragment of the native reductase which was obtained after cleavage of a 10 kDa hydrophobic part of the enzyme,

was unable to reduce cytochrome P-450. Several studies were conducted to modify the SH groups present in the protein. The interaction of cytochrome P-450 and of cytochrome c with the enzyme was studied. The latest investigations were on cloning the gene of the enzyme and to bring the gene to expression.

Chapter 2 deals with a ^{31}P NMR study of the reductase. The ^{31}P spectrum of the enzyme showed besides the three resonances due to the flavins some other phosphate resonances. 2'AMP phosphate was present in every preparation in an almost 1:1 ratio. The amount of phospholipids present in the samples varied. It was demonstrated that these phospholipids were of importance for the configuration of the FMN binding site. ^{31}P NMR spectra of the reductase in the various redox states gave some information on how the environment of the phosphates is influenced by the state of reduction. The semiquinone state of the reductase showed some broadening of the FMN-phosphate resonance due to the paramagnetic free electron only when phospholipids are present in the sample. In the absence of phospholipids no broadening has been observed. The function of these phospholipids has to be studied in more detail. Adding the paramagnetic Mn^{++} ion to the protein showed that the phospholipids are on the surface of the enzyme and that the 2'AMP phosphate is to some extent accessible by the solvent.

In Chapter 3 time-resolved fluorescence studies on the NADPH-cytochrome P-450 reductase are reported. Although the flavins in the reductase are slightly fluorescent it was possible to study the time-resolved fluorescence- and anisotropy decays, which are both multi-exponential. It is shown that FMN in the FMN-reconstituted preparation was not bound in the same way as in the native reductase. It was bound much more loosely although the activity was almost completely regained. Because there is a small spectral overlap between the absorption and emission spectra of FMN and FAD, respectively, a certain degree of energy transfer between the two flavins is possible. We used dimers of methyllumiflavin as models. By measuring the steady state fluorescence anisotropy at different excitation wavelength we discovered that at certain excitation wavelength there was no energy transfer. The same was true for the reductase. By measuring the time-resolved fluorescence anisotropy decay at the wavelength where energy transfer occurs the decay could be fitted by three exponentials, while in the case of no energy transfer the decay could be fitted by two exponentials. The third component, a rather fast one, was

absent in the former case and could be described to energy transfer. With this fast component it was possible to calculate the distance between the FAD and the FMN in the reductase (about 8-13 angstrom).

In Chapter 4 we report on a recombination study of the FMN-depleted reductase *with different modified flavins*. NADPH-cytochrome P-450 reductase was easily made FMN-depleted and could be reconstituted with FMN to an active enzyme again. We studied if some modified flavins were able to do this as well and how fast the rate of recombination was compared to that of the native FMN. It was shown that an extra phenyl group on the 6,7 position of the isoalloxazine moiety of the FMN was of no concern to the recombination rate and the recovery of the activity. An extra phosphate group on the 3' position of the ribityl chain was disastrous to the recovery of the activity; the 3'5'biphosphate riboflavin did not bind at all on FMN-depleted reductase. Other modified flavins did bind and gave recovery of the activity to different degrees.

Chapter 5 describes several procedures to make the FMN-depleted reductase. Further some physical techniques were applied to the native , the FMN-depleted- and the FMN-recombined reductase to see if the procedure changes some physical properties of the enzyme. Because the published procedure to make FMN-depleted reductase was of no use to us (dialysis of the reductase against a buffer containing 2 M KBr), we searched for a better procedure. Binding of the enzyme on a bioaffinity Sepharose column and removal of the FMN by washing with a certain buffer and elution of the FMN-depleted protein, led to loss of FMN and FAD. Therefore another procedure was tested. Dilution in a buffer containing 2 M KBr , concentration of the solution by ultrafiltration and repeating this several times, led to a sample containing small amounts of FMN. A method is described to calculate the percentage of FMN-depleted reductase if one starts with a certain amount of enzyme and a certain number of iterations of the procedure. Large amounts of reductase could not be made FMN-depleted by this procedure. A third procedure (applying the enzym on a Biogel gelfiltration column and eluting the FMN-depleted protein with a buffer containing 2 M KBr) was also not succesfull in obtaining a FMN-free preparation of the enzyme. A combination of the procedures followed by binding of the FMN-depleted enzyme on the 2'5'ADP-Sepharose column, led to a completely FMN-depleted reductase preparation. Certain physical techniques (Circular dichroism, Analytical ultracentrifugation, Time-resolved fluorescence spectroscopy, Steady state fluorescence spectros-

copy and ^{31}P Nuclear magnetic resonance) showed that FMN in the FMN-recombined reductase was bound different from the FMN in the native enzyme. Aggregation of the enzyme occured only if the preparation was frozen at -70°C. The binding of the FAD is influenced by the apo-procedure. Also the conformation of the apo-enzyme is influenced slightly by this procedure as shown by CD.

SAMENVATTING

Het onderzoek naar flavines en flavoproteinen begon in 1879 met de ontdekking van het gele pigment "lactochrome" in wei door Blyt. Later bleek echter dat dit "lactochrome" en enkele andere geisoleerde gele pigmenten derivaten waren van riboflavine (vitamine B_2). Het eerste eiwit dat een dergelijk flavine bevatte was het "old yellow enzyme" NADPH dehydrogenase, wat geisoleerd werd uit gist in 1932. Momenteel zijn zo'n honderd flavoproteinen bekend, welke hetzij FAD en/of FMN as prothetische groep bevatten. De flavine coenzym kan of covalent of niet covalent aan het eiwit gebonden zijn. Hun functie kan daarentegen erg verschillen. Sommige flavoproteinen werken als dehydrogenasen, terwijl andere oxidasen zijn. Müller schreef in 1981 een review over de verschillende flavoproteinen die in de natuur voorkomen.

Het enzyme dat in dit proefschrift behandeld wordt , NADPH-cytochroom P-450 reductase bevat twee flavines : FAD en FMN, welke beide niet covalent gebonden zijn aan het eiwit.

Hoofdstuk 1 bevat een nogal uitgebreid overzicht over dit specifiek flavoprotein en de lezer wordt hiernaar verwezen voor verdere informatie. Een kort overzicht over dit enzyme is hier echter toch op zijn plaats. NADPH-cytochroom P-450 reductase, welke zowel FAD als FMN als prostetische groep bevat, werd als eerste ontdekt door Horecker in 1949 alhoewel hij het zelf niet wist. Later raakten meerdere onderzoekers betrokken bij de isolatie tot een homogeen enzym en bij het bewijs van zijn functie. NADPH-cytochroom P-450 reductase is een membraan gebonden flavoproteine, wat voornamelijk aanwezig in het endoplasmatisch reticulum vam de lever. Het enzyme maakt deel uit van het "mixed function oxidase" systeem en transporteert electronen van NADPH naar cytochroom P-450 door middel van zijn flavines. Het multi-enzyme systeem cytochroom P-450 vervult een belangrijke rol in de detoxificatie van xenobiotica, de activering van procarcinogenen, het steroid- en vetzuurmetabolisme etc.

The essentiele reactie is de hydroxylering door cytochroom P-450. De aminozuur volgorde van het 78 kDa eiwit is bepaald. Bewezen werd dat er een zekere mate van homologie was tussen het reductase en enkele andere flavine eiwitten. De redoxpotentialen vam de individuele flavines zijn berekend. FMN en FAD konden van het eiwit verwijderd worden en het was ook mogelijk om de activiteit te herstellen na reconstitutie van het flavine-vrije eiwit met het FMN of het FAD. Een 68 kDa proteolytisch fragment van het natieve reductase, welke verkregen werd na afsplitsing van een 10 kDa hydrofoob gedeelte van het enzyme, was niet meer in staat om cytochroom P-450 te reduceren. Verschillende experimenten werden uitgevoerd om de in het eiwit aanwezige SH-groepen te modificeren. De interactie van het cytochroom P-450 en van het cytochroom c met het reductase werd bestudeerd. De laatste onderzoeksexperimenten handelem over cloneren van het gen welke codeert voor het reductase en het tot expressie brengen van die clone.

Hoofdstuk 2 handelt over een ^{31}P NMR-studie over het reductase. Het ^{31}P NMR spectrum van het enzym vertoont behalve de drie flavine fosfaat- nog enkele andere phosphorresonanties. 2'AMP fosfaat was aanwezig in elk monster in bijna 1:1 verhouding. De hoeveelheid phospholipiden in het monster varieerde. Bewezen werd dat die phospholipiden van belang waren voor de configuratie van de FMN bindingsplaats. ^{31}P NMR spectra van het reductase in de verschillende redoxtoestanden verschafte informatie hoe de omgeving van de fosfaatgroepen beinvloed wordt door de reductie toestand. De semiquinon toestand van het reductase liet een verbreding van de FMN fosfaat resonantie zien als gevolg van het paramagnetische vrije electron en dit alleen wanneer phospholipiden aanwezig waren in het monster. In de afwezigheid van die phospholipiden werd geen verbreding waargenomen. De rol van die phospholipiden zal daarom nader bestudeerd dienen te worden. Toevoeging van het paramagnetische Mn^{++} ion aan het eiwit toonde aan dat de phospholipiden zich aan de buitenkant van het enzym bevonden en dat de 2'AMP fosfaat tot op bepaalde hoogte toegankelijk is voor het oplosmiddel.

In hoofdstuk 3 worden tijdopgeloste fluorescentie studies aan het NADPH-cytochroom P-450 reductase beschreven. Ofschoon de flavines in het reductase slechts licht fluorescent zijn was het mogelijk de tijdsafhankelijke fluorescentie- en anisotropieverval, welke beide multi-exponentieel zijn, te bestuderen. Aangetoond werd dat FMN in FMN-gereconstitueerd monster niet op dezelfde manier gebonden zat als in het natieve

reductase. Het zat veel losser gebonden ofschoon de activiteit bijna totaal terugkeerde. Omdat er een kleine spectrale overlap bestaat tussen de absorptie- en emmissiespectra van FMN en FAD, respectievelijk, is er enige mate van energie overdracht tussen de twee flavines mogelijk. We gebruikten dimeren van methyllumiflavine als modellen. Door de steady state fluorescentie anisotropie te meten bij verschillende excitatie golflengten ontdekten we dat er bij een bepaalde excitatie golflengte geen energie overdracht aanwezig was. Hetzelfde bleek voor het reductase op te gaan. Door de tijdopgeloste fluorescentie anisotropie verval te meten bij die excitatie golflengte waar energie overdracht optrad, bleek dat het verval gefit kon worden met drie exponenten, terwijl in het geval van geen energie overdracht het verval gefit kon worden met twee exponenten. De derde component, een vrij snelle, was afwezig in het eerste geval en kon worden toegeschreven aan energie overdracht. Met deze snelle component was het mogelijk om de afstand tussen FAD en FMN in het reductase te berekenen (ongeveer 8-13 Å).

In hoofdstuk 4 rapporteren we over een recombinatiestudie van het FMN-vrije reductase met verschillende gemodificeerde flavines. NADPH-cytochroom P-450 reductase kon vrij gemakkelijk FMN-vrij gemaakt worden en kon weer met FMN gereconstitueerd worden tot een actief enzym. Wij bestudeerden of enkele gemodificeerde flavines ook tot dit in staat waren en hoe snel de recombinatie verliep in verhouding tot die van het natieve FMN. Aangetoond werd dateen extra phenyl groep op de 6,7 positie van de iso-alloxazine ring van het FMN niet van invloed was op de recombinatiesnelheid en het herstel van de activiteit. Een extra fosfaatgroep op de 3' positie van de ribitylketen was desastreus voor het herstel van de activiteit; 3'5'bifosfaat ribiflavine bond bijna niet aan FMN-vrij reductase. Andere gemodificeerde flavines bonden wel en gaven tot verschillende mate herstel van de activiteit te zien.

Hoofdstuk 5 beschrijft enkele procedures om het FMN-vrije reductase te maken. Verder werden enkele physische technieken toegepast op het natieve-, het FMN-vrije en het FMN gerecombineerde reductase om te zien of de procedure enkele physische eigenschappen van het enzyme verandert. Omdat de gepubliceerde procedure om FMN-vrij reductase te maken voor ons niet bruikbaar was (dialyse van het reductase tegen een buffer welke KBr bevatte), zochten we naar een betere procedure. Binding van het enzym aan een bioaffiniteitskolom en verwijdering van het FMN door wassen met een bepaalde buffer en elutie van het FMN-vrije eiwit, leidde tot ver-

lies van FMN en FAD. Daarom werd een andere procedure getest. Verdunnen in een 2 M KBr bevattende buffer, concentratie van de oplossing met een Amicon apparaat met een YM-30 filter en herhaling van deze procedure verschillende keren, leidde tot een monster welke een kleine hoeveelheid FMN bevatte. Een methode werd beschreven om het percentage FMN-vrij reductase te berekenen wanneer men start met een bepaalde hoeveelheid enzym en een bepaald aantal iteraties van de procedure. Grote hoeveelheden van reductase konden op deze manier niet FMN-vrij gemaakt worden. Een derde procedure (het enzym op een Biogel gelfiltratie kolom brengen en elutie van het FMN-vrije eiwit met een 2 M KBr bevattende buffer) was ook niet succesvol om een FMN-vrij preparaat van het enzym te verkrijgen. Een combinatie van deze procedures gevolgd door binden van het FMN-vrije reductase aan de 2 5 ADP-Sepharose kolom, leidde tot een compleet FMN-vrij reductase preparaat. Bepaalde physische technieken (Circulair dichroisme, Analytische ultracentrifugatie, Tijdopgeloste fluorescentie spectroscopie, Steady state fluorescentie spectroscopie en ^{31}P-Kernspin magnetische resonantie) lieten zien dat FMN in het FMN-gerecombineerde reductase anders was gebonden dan in het natieve enzym. Aggregatie van het enzym gebeurde alleen als het monster ingevroren was geweest bij -70°C. De binding van het FAD werd beinvloed door de apo-procedure. Ook de conformatie van het apo-enzym werd licht beinvloed door de apo-procedure wat met CD werd aangetoond.

CURRICULUM VITAE

Peter Bonants werd geboren op 5 april 1957 te Venray. In 1975 werd het diploma Gymnasium B behaald aan het Boschveldcollege te Venray. In datzelfde jaar werd een aanvang genomen met de studie scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen werd afgelegd in 1978 en het doctoraalsdiploma werd in 1982 behaald. Het doctoraalprogramma omvatte een uitgebreid hoofdvak op de vakgroep Biochemie (prof. dr.Bonting en prof.dr.Bloemendal) , een bijvak op de vakgroep Toxico-Farmacologie (prof.dr.Henderson) en een bijvak Klinische Chemie op het laboratorium van het St.Radboudziekenhuis (dr.Jansen). Vervolgens werd vrijwilligerswerk verricht bij de Stichting Ziekenhuisapotheek en Klinisch Laboratorium Venray. Gedurende enkele maanden werd als vervangend docent les gegeven aan het Dr.Struyckeninstituut (HLO) te Etten-Leur. In 1984 werd een aanvang genomen met het promotie-onderzoek onder leiding van Prof.dr.F.Müller aan de Landbouwuniversiteit te Wageningen , vakgroep Biochemie.