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Cytochrome b_5, Cytochrome *c*, and Cytochrome P-450 Interactions with NADPH–Cytochrome P-450 Reductase in Phospholipid Vesicles

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ABSTRACT: Upon incubation of detergent-solubilized NADPH–cytochrome P-450 reductase and either cytochrome b_5 or cytochrome *c* in the presence of a water-soluble carbodiimide, a 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), covalently cross-linked complex was formed. The cross-linked derivative was a heterodimer consisting of one molecule each of flavoprotein and cytochrome, and it was purified to 90% or more homogeneity. The binary covalent complex between the flavoprotein and cytochrome b_5 was exclusively observed following incubation of all three proteins including NADPH–cytochrome P-450 reductase, cytochrome b_5, and cytochrome *c* in L-α-dimyristoylphosphatidylcholine vesicles, and no heterotrimer could be identified. The isolated reductase–cytochrome b_5 complex was incapable of covalent binding with cytochrome *c* in the presence of EDC. No clear band for covalent complex formation between PB-1 and reductase was seen with the present EDC cross-linking technique. More than 90% of the cross-linked cytochrome *c* in the purified derivative was rapidly reduced upon addition of an NADPH-generating system, whereas approximately 80% of the cross-linked cytochrome b_5 was rapidly reduced. These results showed that in the greater part of the complexes, the flavin-mediated pathway for reduction of cytochrome *c* or cytochrome b_5 by pyridine nucleotide was intact. When reconstituted into phospholipid vesicles, the purified amphipathic derivative could hardly reduce exogenously added cytochrome *c*, cytochrome b_5, or PB-1, indicating that the cross-linked cytochrome shields the single-electron-transferring interface of the flavoprotein. These results suggest that the covalent cross-linked derivative is a valid model of the noncovalent functional electron-transfer complex.

Hepatic microsomal NADPH–cytochrome P-450 reductase is a membrane-bound flavoprotein which is responsible for electron transfer from NADPH to cytochrome P-450 in the oxidative metabolism of numerous endogenous and foreign compounds (Conney, 1976; Gillette et al., 1972). This reductase is also involved in electron transfer to other heme proteins, such as ferric heme oxidase and hemoglobin (Guengerich, 1978). In addition, the reductase can transfer electrons to cytochrome b_5, and it was recently demonstrated that it may replace NADH–cytochrome b_5 reductase to support NADPH-dependent desaturation of fatty acids (Enoch & Strittmatter, 1979; Daily & Strittmatter, 1980). It is anchored to the microsomal membrane by its hydrophobic amino-terminal region (Black et al., 1979; Gum & Strobel, 1981). This hydrophobic tail, which is readily cleaved from the intact protein by various proteases including trypsin, is essential for the proper interaction of reductase with either cytochrome P-450 (Black et al., 1979; Gum & Strobel, 1981) or cytochrome b_5 (Enoch & Strittmatter, 1979). However, a flavoprotein which loses its tail by protease digestion retains the ability to reduce cytochrome *c* (Masters et al., 1975; Lu et al., 1969). In fact, proteolysis of the cross-linked covalent

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complex containing one molecule each of the flavoprotein and cytochrome b_5 indicated that the hydrophilic catalytic domains participate in the covalent attachment and that the hydrophobic membrane-anchored peptide is necessary for protein interactions (Hackett & Strittmatter, 1984; Nisimoto & Lambeth, 1985).

Electron transfer between flavoprotein and cytochrome is fundamental to many biochemical pathways, such as mitochondrial and microsomal electron-transfer chains. Thus, an understanding of the mechanism of interactions between NADPH–cytochrome P-450 reductase and cytochrome P-450 or cytochrome b_5 in a reconstituted phospholipid vesicle may help to define the location of the cytochrome binding domain on the flavoprotein molecule. Most electron-transfer complexes are stable at low ionic strength, so certain complexes can be cross-linked by some bifunctional chemical reagent. The water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC)[1] has proven useful for the covalent cross-linking of charge-paired functional protein–protein complexes (Lambeth et al., 1979; Millet et al., 1983; Hackett & Strittmatter, 1984).

Our earlier studies with this flavoprotein (Nisimoto & Lambeth, 1985; Nisimoto, 1986) indicated that EDC provides a specific formation of amide bonds between complementary charge pairs during the interaction of membrane-bound reductase and either cytochrome b_5 or cytochrome c. Thus, the line of approach in the present study has been to purify and characterize the reactive covalent complex, which presumably utilizes charge pairing between the flavoprotein and one of its physiological electron acceptors such as PB-1, cytochrome b_5, or cytochrome c in the plane of phospholipid membranes. In addition, our present study has shown that the FMN domain on reductase is essential for the interaction not only with P-450 or cytochrome c (Vermilion & Coon, 1978) but also with cytochrome b_5 during redox reaction. Using the purified covalent binary complex between the reductase and cytochrome b_5 (or cytochrome c), we also report on the mechanism and stoichiometry of protein–protein interactions in the cytochrome b_5 participating mixed-function oxidase system of rat liver.

Experimental Procedures

Materials. NADPH, FAD, FMN, cytochrome c, Triton N-101, deoxycholate, L-α-dimyristoylphosphatidylcholine (DMPC), EDC, and N,N'-dicyclohexylcarbodiimide were purchased from Sigma Chemical Co. DEAE-Sepharose CL-6B, 2′,5′-ADP–Sepharose 4B, and Sephadex G-100 were obtained from Pharmacia. Rat NADPH–cytochrome P-450 reductase was purified from phenobarbital-treated rat liver by DEAE ion-exchange, 2′,5′-ADP–Sepharose 4B, and hydroxylapatite (Bio-Rad) column chromatographies (Iyanagi & Mason, 1973; Yasukochi & Masters, 1976). The NADPH–cytochrome c reductase activity of the purified preparation was about 36 μmol of cytochrome c reduced min^{-1} $(mg\ of\ protein)^{-1}$. The reductase concentration was determined spectrophotometrically from the flavin absorbance at 456 nm (French & Coon, 1979), using an extinction coefficient of 11.5 $mM^{-1}\cdot cm^{-1}$. Detergent-solubilized cytochrome b_5 was purified from rat liver microsomes as described by Ito and Sato (1968) to a specific heme content of 45 nmol/mg of protein. The cytochrome b_5 concentration was calculated from the reduced minus oxidized absorption difference at 423 and 409 nm, using a difference extinction coefficient of 185 $mM^{-1}\cdot cm^{-1}$ (Omura & Sato, 1964). The purification of cytochrome P-450 was performed according to the method of Imai and Sato (1974). The major species of cytochrome P-450 (PB-1) in liver microsomes of phenobarbital-treated rats was isolated to a specific content of 14 nmol of heme/mg of protein. The PB-1 concentration was calculated from the reduced carbon monoxide difference spectrum ($\Delta A_{450-490nm}$) using the extinction coefficient of 91 $mM^{-1}\cdot cm^{-1}$ (Omura & Sato, 1964). This protein electrophoresed as one major band of M_r 54K and one minor band (M_r 52K) on a polyacrylamide gel run in the presence of SDS under the conditions described (Laemmli, 1970).

Cross-Linking of NADPH–Cytochrome P-450 Reductase and either Cytochrome b_5, Cytochrome c, or PB-1 in Phospholipid Vesicles. Detergents in the purified samples were removed by hydroxylapatite chromatography and dialysis prior to reaction, and potassium ion was simultaneously replaced by sodium ion by these procedures. PB-1 was incubated (1 h, 25 °C) with small, unilamellar DMPC liposomes (3.5 μmol of PC) in 20 mM MES buffer, pH 6.4. NADPH–cytochrome P-450 reductase and solid NADPH (2.5 mM) were added to the liposomes containing PB-1 and incubated for 1 h at 25 °C. The reductase:PB-1:DMPC molar ratio in the proteoliposomes was $1:10:10^4$. Thus, the phospholipid concentration was sufficiently high to avoid problems of protein aggregation.

Previous results (Strittmatter & Rogers, 1975) have shown that this reconstitution procedure leads to complete binding of each protein to the vesicles. Cross-linking between the two proteins was carried out in the presence of 5 mM EDC according to the methods described (Hackett & Strittmatter, 1984). The cross-linking reaction was performed at room temperature for 20 min with slow stirring in 20 mM MES buffer, pH 6.4, containing 10% glycerol. Small amounts of reaction mixture (30 μL) were added to 15 μL of dissociation buffer (1% SDS, 0.5% β-mercaptoethanol, 0.4 M sodium phosphate buffer, and 0.01% bromphenol blue), mixed by vortexing, and heated for 10 min at 100 °C for subsequent analysis by SDS–polyacrylamide gel electrophoresis as described (Rudolph & Krueger, 1976). Approximately 10–30 μg of protein was used for each gel lane, and after visualization of the cross-linking band, the remaining bulky amount of reaction mixture was subjected to the isolation of cross-linked amphipathic derivative. Cross-linking of the reductase and cytochrome b_5 or (and) cytochrome c in DMPC liposomes was carried out in the presence of 5 mM EDC, according to the methods described in previous papers (Nisimoto & Lambeth, 1985; Nisimoto, 1986).

Purification of Cross-Linked Amphipathic Derivative. Following the cross-linking reaction, purification of the covalent cross-linked complex between the reductase and cytochrome b_5 (or cytochrome c) was performed as before (Nisimoto & Lambeth, 1985; Nisimoto, 1986) except that hydroxylapatite chromatography was used in a final purification step in the present study. The finally purified cross-linked derivative was analyzed for purity, monomeric molecular weight, and stoichiometry of cytochrome and reductase in the complex. The total cytochrome b_5 concentration in the cross-linked derivative was calculated by the method of Omura and Sato (1964). Fluorophotometric assay of total flavin was performed on the neutralized supernatant following precipitation of the protein and liberation of flavin using 20% trichloroacetic acid. Fluorescence measurements were carried

[1] Abbreviations: PB-1, rat liver microsomal cytochrome P-450 induced by phenobarbital; P-450LM_2, rabbit liver microsomal P-450 induced by phenobarbital; DMPC, L-α-dimyristoylphosphatidylcholine; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DCPI, dichlorophenolindophenol; SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; FMN, flavin mononucleotide; MES, 2-(N-morpholino)-ethanesulfonic acid; FAD, flavin adenine dinucleotide; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane.

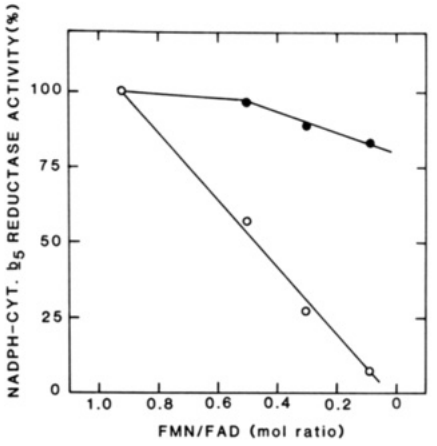

FIGURE 1: Effect of FMN on NADPH–cytochrome b_5 reductase activity. Several KBr-treated reductase preparations were pooled and finally dialyzed against 0.05M Tris–acetate buffer, pH 7.7, containing 0.1% Triton N-101 and 10% glycerol. The molar ratios of FMN to FAD in the preparations were 0.51, 0.30, and 0.09, respectively. The rates of cytochrome b_5 reduction in the presence (closed circle) and absence (opened circle) of added FMN (0.01 mM) were measured in 0.05 M phosphate buffer, pH 7.2, at 30 °C. All assay mixtures containing 4.1 μM (or 2.3 μM) reductase, 78.4 μM cytochrome b_5, 3.5 mM DMPC, 1.5 mM DOC, and either the absence or the presence of added FMN were preincubated for 30 min at 25 °C, and the reaction was initiated by the addition of 0.1 mM NADPH. The changes of cytochrome b_5 reductase activity were indicated in percentage by using native enzyme activity [62 nmol of cytochrome b_5 reduced min^{-1} (mg of protein)$^{-1}$] regarded as 100%.

out using a Hitachi fluorescence spectrophotometer, Model 650-60, with an excitation wavelength of 450 nm and an emission wavelength of 525 nm. Fluorescence intensity was measured at both pH 7.7 and pH 2.6, thus allowing calculation of both FAD and FMN contents (Faeder & Siegel, 1973). The isolation of the covalent cross-linked derivative composed of reductase and cytochrome *c* was also carried out according to the methods we have described before (Nisimoto, 1986), except for some modification to raise the yield.

Preparation of FMN-Depleted NADPH–Cytochrome P-450 Reductase. FMN-depleted enzyme was prepared by the same method described by Vermilion and Coon (1978). The preparation of FMN-depleted reductase obtained by this procedure contained 95% or more of the FAD present in the native enzyme but approximately 10–55% of the original amount of FMN. The NADPH–cytochrome *c* (or P-450) reductase activity corresponded quite closely to the amount of FMN remaining in the preparation.

Measurement of Protein and Activities. Protein was assayed by the method of Lowry et al. (1951) using bovine serum albumin as standard. Enzymatic activities of the reductase and purified cross-linked derivative were measured at 25 °C in 50 mM potassium phosphate buffer, pH 7.2. The rate of cytochrome *c* reduction by NADPH was followed by using absorbance changes at 550 nm, with the extinction coefficient of 18.5 $mM^{-1}\cdot cm^{-1}$ (Van Gelder & Slager, 1962). Cytochrome b_5 reductase activity was determined from the absorption changes, using an extinction coefficient of 100 $mM^{-1}\cdot cm^{-1}$ at 424 nm (Strittmatter et al., 1972) in the presence of DMPC liposomes. PB-1 reductase activity is followed in the presence of 1 mM benzphetamine and phospholipid and expressed as micromoles of NADPH oxidized per minute per milligram of protein.

RESULTS

FMN-Dependent Reductase Activity with Cytochrome b_5. It was reported that dialysis of flavoproteins against high

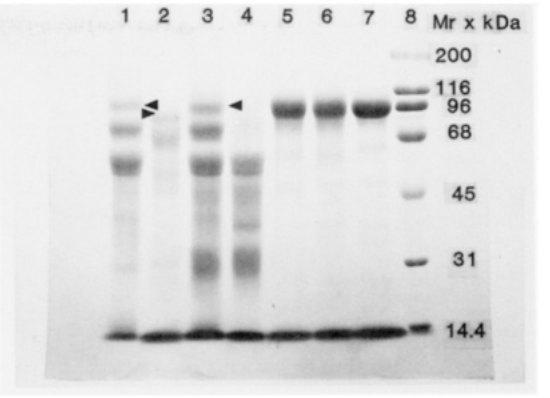

FIGURE 2: Cross-linking of NADPH–cytochrome P-450 reductase, cytochrome b_5, and cytochrome *c* in phospholipid vesicles. Reconstituted proteoliposomes composed of the following proteins and 2.5 μmol of DMPC were incubated with 0.1 mM NADPH in 20 mM MES buffer, pH 6.4, containing 10% glycerol at 25 °C for 10 min, and then 5 mM EDC was reacted with the incubated mixture at 36 °C for 15 min. Lanes 1–7 show SDS gel electrophoretic patterns of the proteoliposomes after the EDC cross-linking reaction: lane 1, 2.35 nmol of reductase (as protein) plus 14.1 nmol of cytochrome b_5 (as heme); lane 2, 2.35 nmol of reductase plus 10.5 nmol of cytochrome *c* (as heme); lane 3, 2.35 nmol of reductase plus 14.1 nmol of cytochrome b_5 plus 10.5 nmol of cytochrome *c*; lane 4, 14.1 nmol of cytochrome b_5 plus 15.5 nmol of cytochrome *c*. Lanes 5–7 show the reaction at a molar ratio of cytochrome *c* to reductase–cytochrome b_5 complex (2.68 nmol as protein) of 1.9, 3.9, and 5.8, respectively. The last gel lane shows marker proteins which are myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme whose apparent molecular weights are 200 000, 116 000, 96 000, 68 000, 45 000, 31 000, and 14 400, respectively. Arrows point to reductase–cytochrome b_5 dimer (◀) and reductase–cytochrome *c* dimer (▶).

concentrations of KBr is a mild procedure frequently used to obtain apoenzyme preparations suitable for reconstitution studies (Vermilion & Coon, 1978). As shown in Figure 1, significant dissociation of FMN from NADPH–cytochrome P-450 reductase was observed in the presence of 2 M KBr at pH 7.0. However, loss of FAD was minimal under the conditions used, and the ability of the enzyme to interact with pyridine nucleotide was not significantly altered by FMN removal. The three preparations of FMN-depleted reductase used in the present experiments contained more than 95% of FAD present in the native reductase but only 10–55% of the original amount of FMN. Consistent with the earlier studies (Vermilion & Coon, 1978) with P-450LM_2, cytochrome *c*, menadione, and DCPI, the activity of the FMN-depleted reductase was very low, but the ability to reduce these electron acceptors was nearly completely restored after incubation with added FMN (data not shown).

Thus, these results suggested the direct involvement of reduced FMN to interact with these acceptors. Similarly, the FMN-depleted preparation lost the activity for the reduction of cytochrome b_5. The relative activity corresponded closely to the amount of FMN remaining in the preparation, and when the FMN-depleted reductase was assayed after incubation with FMN, NADPH–cytochrome b_5 reductase activity was elevated to approximately that seen with the native enzyme.

It is concluded from these results that not only the reactions with cytochrome P-450 and cytochrome *c* but also the interaction with cytochrome b_5 proceeds via an FMN-dependent pathway and that the FAD domain is essential to accept electrons from NADPH.

Covalent Cross-Linking of Vesicle-Bound NADPH–Cytochrome P-450 Reductase and either Cytochrome b_5, Cytochrome c, or PB-1. As shown in Figure 2, the formation of cross-linked protein species composed of cytochrome b_5 and

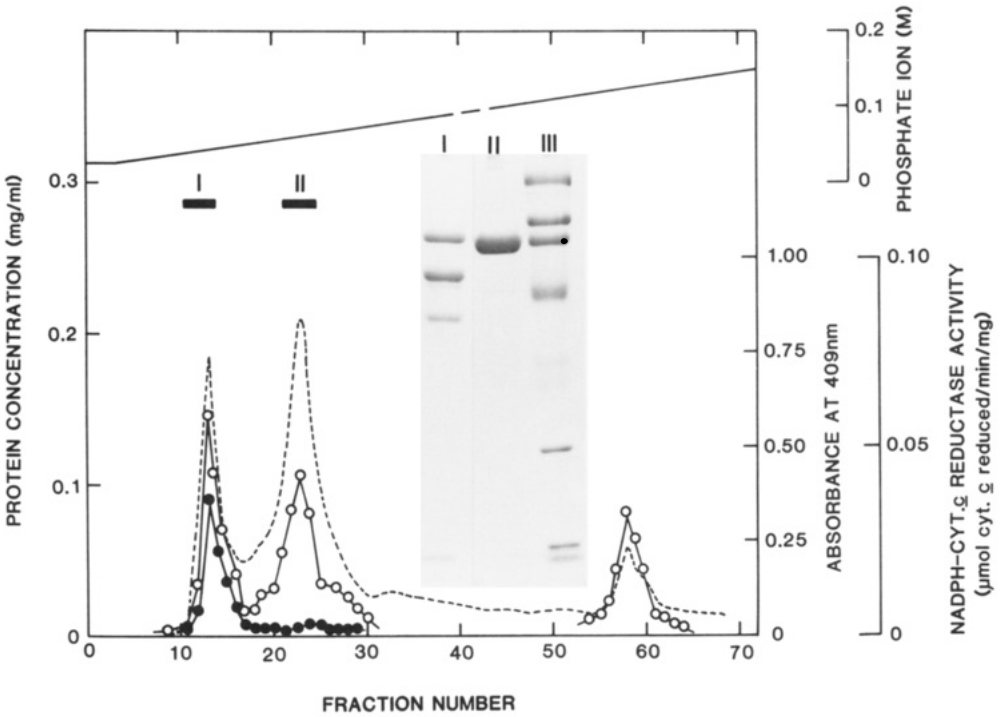

FIGURE 3: Separation of the cross-linked derivative from unreacted reductase on a hydroxylapatite column. Pooled fractions from the second 2′,5′-ADP–Sepharose chromatography were loaded onto a hydroxylapatite column (1.5 × 7 cm) equilibrated with 25 mM phosphate buffer, pH 7.0, containing 20% glycerol and 0.4% DOC. The column was washed successively with a linearly increasing concentration of potassium phosphate buffer, pH 7.0 (0.025–0.20 M). The flow rate was 6 mL/h, and fractions of 2 mL were collected. Aliquots from the pooled peak fractions indicated by horizontal bars (I and II) were concentrated and analyzed by SDS gel electrophoresis. Gel lane III shows the same marker proteins as used in Figure 2. The protein (dashed line), cytochrome b_5 concentration (open circles), and NADPH–cytochrome *c* reductase activity (closed circles) were measured as described under Experimental Procedures.

NADPH–cytochrome P-450 reductase was observed after addition of EDC. The molecular size of this new, rapidly formed species was estimated from a plot of molecular weight vs mobility to be 93K, a value consistent with a 1:1 cross-linked complex of cytochrome b_5 plus reductase (lane 1). In addition, a 1:1 cross-linking complexed band was also seen after amphipathic native reductase and cytochrome *c* were incubated with small unilamellar DMPC vesicles in the presence of EDC (lane 2). No clear SDS gel band of a size corresponding to a 1:1 cross-linked complex between PB-1 and reductase could be observed under the conditions used (data not shown), although a very weak chemical cross-linking band of rabbit reductase plus P-450LM$_2$ has been demonstrated by Tamburini et al. (1986).

When reductase was preincubated with both cytochrome b_5 and cytochrome *c* in the presence of EDC, a new strongly staining protein band appeared in subsequent SDS gel electrophoresis with a molecular weight value (93K) close to a binary covalent complex of cytochrome b_5 and reductase (lane 3). However, neither a distinct cross-linked species between reductase and cytochrome *c* nor a heteroternary covalent complex formation composed of the three proteins was identified. Unlike reductase or cytochrome *c*, when cytochrome b_5 was incubated with cytochrome *c* in the presence of EDC, cytochrome b_5 yielded significant amounts of cross-linked protein with molecular weight values of 57K, 52K, and 33K which are probably ascribed to most cytochrome b_5 homodimer and homotrimer and to a small amount of heterodimer and heterotrimer containing both the cytochromes (lane 4). Incubation of the purified reductase–cytochrome b_5 covalent complex with cytochrome *c* in the presence of EDC did not give rise to an additional protein band for a ternary complex (apparent M_r 107K) composed of the three components, even if cytochrome *c* concentration was increased in the reconstituted proteoliposomes (lane 5–7).

Purification and Properties of Cross-Linked Complex Composed of Cytochrome b_5 and NADPH–Cytochrome P-450 Reductase. The buffer, pH, reaction time, and cytochrome:reductase:DMPC molar ratio in liposomes were optimized by monitoring the extent of cross-linking under conditions with SDS gel electrophoresis. After EDC cross-linking as described under Experimental Procedures, the purification of the 93-kDa cross-linked derivative was undertaken to confirm its identity and characterize its properties. Following cross-linking, the complex was purified by a combination of affinity chromatography on 2′,5′-ADP–Sepharose 4B and DEAE-Sepharose CL-6B and Sephadex G-100 chromatography according to the method described (Nisimoto & Lambeth, 1985). In order to raise the purity and yield, hydroxylapatite column chromatography was also used in a final purification step (Figure 3). As seen in the SDS gel electrophoresis pattern (inset in Figure 3), the pooled fraction II contains only the single homogeneous cross-linked protein (93-kDa band), and a small quantity of free reductase (77-kDa band) was found only in the earlier fraction peak I and coincided with residual NADPH–cytochrome *c* reductase activity. The yield of the cross-linked derivative following reaction and purification was low (approximately 6.5%) compared with the original amount of reductase used. The pooled fraction peak II was concentrated and used to characterize the isolated cross-linked derivative.

Eighty percent or more of the covalently bound cytochrome b_5 in the purified complex can be rapidly reduced within a few seconds by addition of an NADPH-generating system, and the rest is reduced by dithionite (Figure 4). Changes in the reduction state of flavin were also quantitated by using absorbance changes at 439 nm (an isosbestic point for cytochrome b_5). In agreement with these results, about 80–90% of the dithionite-reducible flavin was reduced rapidly by NADPH. Thus, the present results indicate that most of the

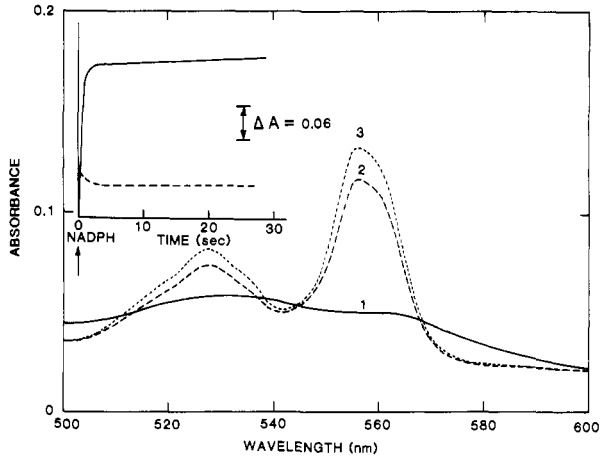

FIGURE 4: Reduction of cytochrome b_5 in the covalently cross-linked derivative by an NADPH-generating system. The oxidized form (4.8 μM heme, spectrum 1) in 0.05 M potassium phosphate buffer, pH 7.2, was reduced in the presence of 0.1 mM NADPH, 1 mM glucose 6-phosphate, and 5 units of glucose-6-phosphate dehydrogenase at 20 °C. Curve 2 shows the absorption spectrum measured immediately after the addition of NADPH. The experimental curve was not corrected for dilution. Spectral curve 3 indicates a fully reduced state after reduction with a trace amount of solid dithionite. The inset represents the rates for the reductions of covalently bound cytochrome b_5 (solid line) and flavin (dashed line) in the complex. They were followed by the absorption changes at 424 and 439 nm immediately after the addition of NADPH.

bound cytochrome can accept electrons from NADPH via flavins in the complex. About 20% of the dithionite-reduced material present may represent a combination of complexes cross-linked in nonoptimal conformations and significant inactivation of the reductase during the preparation and purification of the cross-linked population. The estimation of the rate constant of rapid reduction was not attempted in the present study.

As shown in Figure 5, electron acceptor (cytochrome c and cytochrome b_5) reductase activities of the purified derivative were greatly inhibited (less than 10% of native reductase). Furthermore, the rate of NADPH oxidation in the presence of benzphetamine was enhanced in parallel with increasing concentrations of PB-1 and reductase which were reconstituted into phospholipid membranes; however, the benzphetamine N-demethylation activity was almost completely lost when the purified complex was added instead of native reductase (Figure 6). This lack of reduction may be ascribed to the block of electron transfer from the covalent complex to the exogenously added electron acceptors, because most of the population of the covalently bound cytochrome b_5 was reduced at a rate approaching the maximum velocity of reductase upon addition of NADPH. Thus, our conditions used for the preparation and isolation of derivative did not appear to alter the reductase activity, suggesting that the covalently cross-linked complex is a valid model of the noncovalent electron-transfer complex.

The overall cytochrome b_5 to reductase stoichiometry in the complex can be calculated from the absorption spectrum of the purified derivative and fluorophotometric quantitation of total flavin. On the basis of this calculation, a total flavin to heme ratio was 2.4 in cytochrome b_5–reductase covalent complex (Table I). This result is basically consistent with a 1:1 stoichiometry of cytochrome to reductase in the purified cross-linked complex, since nearly equimolar FAD and FMN are found both in reductase and in the cross-linked complex. On the other hand, the isolated reductase–cytochrome c covalent complex also demonstrated almost the same properties as the reductase–cytochrome b_5 cross-linked derivative in terms of ability to reduce covalently bound cytochrome and externally

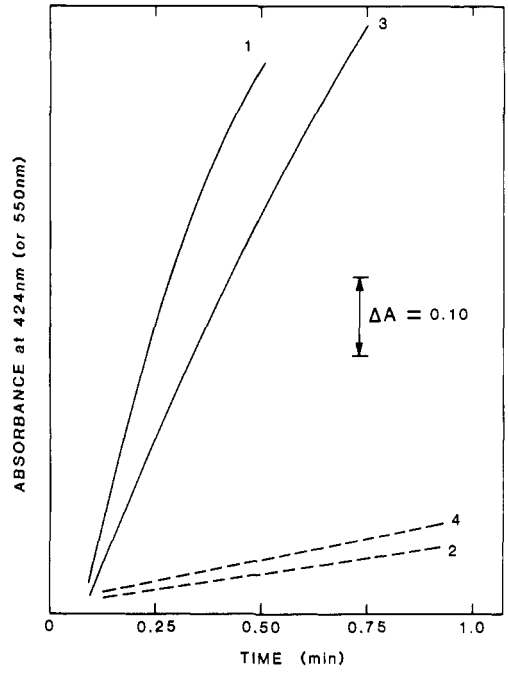

FIGURE 5: Time course of NADPH-dependent cytochrome b_5 (or cytochrome c) reduction either by free NADPH–cytochrome P-450 reductase or by the reductase–cytochrome b_5 cross-linked derivative in DMPC vesicles. Reconstitution was carried out by incubating 4.7 μM free reductase (or 5.5 μM purified covalent complex), 88 μM cytochrome b_5, 3 mM phospholipid vesicles, and 0.2 mM DOC in 0.05 M phosphate buffer, pH 7.2 at 30 °C, for 1 h. The incubated mixtures were then diluted to 8.8 μM cytochrome b_5, and reduction was followed at 424 nm after addition of 0.1 mM NADPH. Curve 1, free reductase; curve 2, covalently complexed derivative. Cytochrome c reduction was followed by the absorption change at 550 nm at 30 °C in mixtures containing 0.094 μM free reductase (or 0.12 μM covalent complex), 0.1 mM cytochrome c, and 0.05 M phosphate buffer, pH 7.2. The reaction was started by addition of 0.1 mM NADPH. Curve 3, free reductase; curve 4, covalent complex.

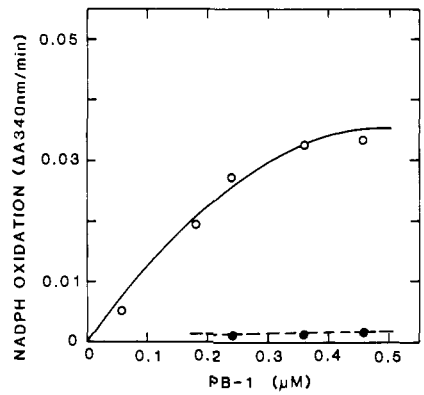

FIGURE 6: Benzphetamine N-demethylation activity by PB-1 incubated either with free reductase or with reductase–cytochrome b_5 complex. The preincubated mixture contained 0.05 M phosphate buffer, pH 7.2, 0.2 μM reductase (○) [or 0.38 μM covalent complex (●)], 1 mM benzphetamine, 50 μM DMPC vesicles, and the indicated amounts of P-450. The reaction was started by adding 0.1 mM NADPH at 30 °C, and the activity was expressed by an absorption change at 340 nm/min.

added electron acceptors (Table I). These results suggest that the functional complex between the reductase and cytochrome in phospholipid vesicles may depend primarily on a single heme protein binding domain on the flavoprotein molecule.

Discussion

As shown in Figure 7, the sophisticated molecule of NADPH–cytochrome P-450 reductase mostly consists of a

Table I: Amphipathic Derivative Composed of NADPH–Cytochrome P-450 Reductase and Cytochrome Isolated after Cross-Linking Reaction with EDC[a]

cyt	cross-link with reductase	flavin:heme mole ratio	app M_r of complex[b]	extent of reduction of bound cyt (%)[c]	electron acceptor activity		
					cyt *c*	cyt b_5	PB-1
b_5	+ (1:1)	2.4	93 000	80	0.88	0.003	0
c	+ (1:1)	1.7	89 000	90	0.92	0.007	0
b_5 and *c*	+ (1:1)		93 000				
PB-1	–						

[a] Cytochrome b_5 and cytochrome *c* reductase activities are expressed as micromoles of acceptor reduced per minute per milligram of protein of complex, and P-450 reductase activity is shown as micromoles of NADPH oxidized per minutes per milligrams of protein. [b] The size of cross-linked complex was estimated from a plot of molecular weight versus mobility in SDS–polyacrylamide gel electrophoresis. [c] Covalent-bound cytochrome b_5 (or cytochrome *c*) in the derivative was reduced in the presence of an NADPH-generating system containing 0.1 mM NADPH, 1 mM glucose 6-phosphate, and 5 units/mL glucose-6-phosphate dehydrogenase at 25 °C. The extents of cytochrome reduced by NADPH were indicated as a percentage of the fully reduced state after dithionite addition.

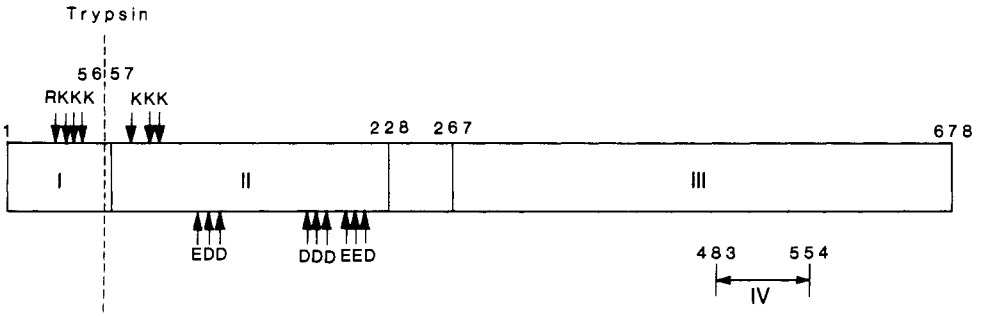

FIGURE 7: Proposed schematic presentation of four structural domains of rat hepatic NADPH–cytochrome P-450 reductase. I, membrane domain; II, FMN domain; III, FAD domain; IV, NADPH domain. The vertical arrows (↓) and (↑) represent cationic and anionic amino acid residues, respectively, which probably participate in charge pairing with cytochrome b_5, cytochrome *c*, and P-450 in phospholipid membranes.

variety of structural domains which may represent gene fusion of polypeptides with simple function, i.e., membrane-anchoring (I), FMN binding (II), FAD binding (III), and NADPH binding domains (IV). Our understanding of the relationships between reductase and function requires the determination of the total structure and the identification of some functional amino acid residues (Lumper et al., 1980; Nisimoto & Shibata, 1982; Haniu et al., 1984). There have been reports that the FMN prosthetic group binding domain is the active center for the reaction with P-450, cytochrome *c*, and cytochrome b_5 by assuming that partially or fully reduced FMN domain interacts with these electron acceptors during catalytic function. Several acidic and basic multiply charged residues are frequently observed in N-terminal and FMN binding regions of rat reductase (Porter & Kasper, 1985): Glu-Asp-Asp (residues 142, 144, and 147), Asp-Asp-Asp (residues 207–209), Glu-Glu-Asp (residues 213–215), Arg-Lys-Lys-Lys (residues 45–48), and Lys-Lys-Lys (residues 72, 74, and 75).

Our earlier study located an amide linkage between Lys-13 of cytochrome *c* and one of the above acidic residues (the 207–215 cluster containing carboxyl groups of Asp and Glu residues) on the flavoprotein. These cytochrome *c* interacting acidic clusters (residues 207–215) were likely to be adjacent to the second FMN binding region (residues 165–202) (Nisimoto, 1986). Furthermore, in the present study, the covalently cross-linked complex composed of reductase and cytochrome *c* almost completely blocked electron transfer from NADPH to exogenous cytochrome *c*, cytochrome b_5, or PB-1 (Table I), suggesting that the cross-linked cytochrome *c* covers the electron-transferring interface on the reductase and the covalent complex is a valid model of noncovalent electron-transfer complex. Porter and Kasper (1986) and Haniu et al. (1986) have also suggested that the regions containing these acidic residues on reductase are very likely candidates for electrostatic complementary charge pairing during the formation of an electron-transfer complex between reductase and either cytochrome *c* or cytochrome P-450.

It is also reported that hydrophobic–hydrophobic interactions between NADPH–cytochrome P-450 reductase and P-450 (or cytochrome b_5) have a significant importance besides charge pairing, since the reductase-lost membrane binding fragment by limited tryptic digestion can neither form a cross-linked complex with cytochrome b_5 (Nisimoto & Lambeth, 1985) nor reduce it in phospholipid vesicles (Enoch & Strittmatter, 1979). According to a hydropathy profile calculated by Kyte and Doolittle (1982), the N-terminal peptide (residues 1–56) and two regions (residues 125–140 and 520–544) apparently exhibit significant hydrophobicity, suggesting membrane, cytochrome b_5, or P-450 interacting domains. These strongly hydrophobic regions as well as the charge effect on reductase may well have crucial importance for interaction with hydrophobic domains of P-450 deeply embedded in the phospholipid bilayer (Lu & Coon, 1968). Thus, it may be considered that the region of PB-1, which is supposed to interact with a vicinity of the FMN binding domain on reductase, is surrounded by hydrophobic circumstances and cannot easily access the hydrophilic EDC–carboxyl adduct located closely to the flavin site of the flavoprotein.

Previous modification studies have shown that the exposed side chain carboxyl groups of Glu-47, -48, and -52 on cytochrome b_5 are commonly involved in protein–protein interaction with NADH–cytochrome b_5 reductase (Dailey & Strittmatter, 1979), cytochrome *c* (Salemme, 1976), or P-450 (Tamburini et al., 1985). These results suggest that complementary charge–charge pairing between carboxyl groups of Glu and Asp residues on cytochrome b_5 and positively charged lysyl residues on NADH–cytochrome b_5 reductase, cytochrome *c*, or P-450 occurs during electron transfer. On the basis of the ternary structure of cytochrome b_5, from the present findings we also suggest probable charge pair interaction of basic residues on NADPH–cytochrome P-450 reductase with the side chain carboxyl groups of acidic residues and the exposed heme propionate on cytochrome b_5. On the basis of the binding of the reductase–cytochrome b_5 covalent

complex to 2′,5′-ADP–Sepharose or NADP–agarose, the bound cytochrome b_5 does not appear to interfere significantly with pyridine nucleotide binding. Also, cytochrome b_5 in the covalent derivative is reducible by NADPH, indicating that the NADPH binding site for initial reduction is not blocked by the bound cytochrome.

However, like the reductase–cytochrome *c* covalent complex, the isolated cross-linked derivative between reductase and cytochrome b_5 almost completely inhibits electron transfer from NADPH to externally added electron acceptors such as cytochrome b_5, cytochrome *c*, and PB-1 (Figures 5 and 6 and Table I). Although other explanations (i.e., binding-induced conformational changes) cannot be rigorously ruled out, the simplest interpretation of these data is that the binding of cytochrome b_5 to the reductase blocks the electron-transfer site(s) on the flavoprotein, thus inhibiting these activities. In addition, a distinct heterotrimer complex could not be observed following incubation of all the above three proteins together with EDC, and cross-linking reaction between the purified reductase–cytochrome b_5 covalent complex and cytochrome *c* in DMPC vesicles did not give an additional band of protein with an approximate molecular weight of 107K for a ternary complex (Figure 2), showing that formation of the reductase–cytochrome b_5 heterodimer blocks the cytochrome *c* interacting domain on the reductase molecule.

It is also suggested that PB-1 is equally inaccessible to direct interaction with the cytochrome binding domain on reductase of the covalent complex, because no P-450 reductase activity was observed in the reaction system containing cytochrome b_5–reductase complex, PB-1, and DMPC vesicles (Figure 6, Table I). Therefore, the P-450, cytochrome *c*, and cytochrome b_5 binding site(s) near the FMN domain is(are) likely to be in very close proximity and probably has(have) elements in common in their charge–charge interactions.

On the basis of these results, in the cytochrome b_5 mediated mixed-function oxidase reaction, we have postulated an electron-transfer model based on binary complex formation between reductase, cytochrome b_5, and PB-1, which appear to undergo relatively free lateral movement in the plane of the membrane so as to permit functional association and dissociation of heterodimer. We assume that several multiply charged clusters of amino acid residues on the FMN domain mentioned previously may be equally important for the electrostatic interaction with the protein surface surrounding the heme group of each cytochrome. Thus, the electron-transferring interface on reductase appears to be impossible to interact simultaneously with both PB-1 and cytochrome b_5 in the mixed-function oxidase reaction. Most recently, Tamburini and Schenkman (1987) have purified an EDC-mediated covalent complex between cytochrome b_5 and P-450LM$_2$ which was reduced by NADPH–cytochrome P-450 reductase, and the complex gave a high mixed-function oxidase activity in the presence of the flavoprotein. From these observations, they have proposed a ternary electron-transfer complex composed of P-450LM$_2$, cytochrome b_5, and NADPH–cytochrome P-450 reductase in which P-450LM$_2$ possesses two separate binding domains to the other two proteins in the redox reaction. This report appears to be incompatible with our present results in which no direct evidence for supporting ternary complex formation could be observed during mixed-function oxidation. Thus, we would need a different experimental approach to investigate the difference in the oxidase reaction mechanisms between microsomal P-450 isozymes.

Registry No. DMPC, 18194-24-6; cytochrome P-450, 9035-51-2; cytochrome *c*, 9007-43-6; cytochrome b_5, 9035-39-6; NADPH–cytochrome P-450 reductase, 9039-06-9.

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The Electron-Transfer Site of Spinach Plastocyanin†

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ABSTRACT: Two sites for electron transfer have been proposed for plastocyanin: one near the copper ion and the other close to the acid patch formed by residues 42–45. Calculations of electrostatic properties of spinach plastocyanin and ionic strength dependences of electron-transfer reactions of this protein have been used to distinguish between these two sites. Calculations show that the electric potential field of spinach plastocyanin is highly asymmetric and that the protein has a dipole moment of 360 D. The negative end of the dipole axis emerges between the negative patches formed by residues 42–45, which is thought to be the cation binding site, and residues 59–61. The angles between the dipole vector and vectors from the center of mass to the copper ion and to the acid patch are 90° and 30°, respectively. The angle between the dipole vector and a line from the center of mass to the site of electron transfer is evaluated from the ionic strength dependence of electron-transfer rates at pH 7.8 with the help of equations developed by Van Leeuwen et al. [van Leeuwen, J. W., Mofers, F. J. M., & Veerman, E. C. I. (1981) *Biochim. Biophys. Acta 635*, 434] and Van Leeuwen [van Leeuwen, J. W. (1983) *Biochim. Biophys. Acta 743*, 408]. The angles found are 85°, 110°, and 75 ± 15° for reactions with tris(1,10-phenanthroline)cobalt(III), hexacyanoferrate(III), and ferrocytochrome *c*, respectively. The electric potential field calculations suggest that the hexacyanoferrate(III) interaction angle corresponds to a unique site of minimum repulsion at the hydrophobic region of the protein surface, close to the copper ion. An interaction angle of 65 ± 15° was determined for (4-carboxy-2,6-dinitrophenyl)lysine 13 ferrocytochrome *c*. The angles found indicate that electron transfer could take place within the hydrophobic region of plastocyanin near the copper ion, possibly via His 87, but not at Tyr 83 or the negative patches. The ionic strength dependence of the association constant between hexaamminecobalt(III) and cuprous plastocyanin was consistent with cation binding near the negative patch formed by residues 42–45.

Plastocyanins are copper-containing proteins that were first discovered in green algae by Katoh (1960). The function of the protein is to carry electrons from cytochrome *f* in photosystem II to pigment $P700^+$ in photosystem I (Anderson, 1982). Recently, the spatial structure of poplar plastocyanin has been determined at 1.6-Å resolution (Guss & Freeman, 1983). It shows that the protein, which consists of a single peptide chain of 99 residues, has a cylindrical shape. Due to an excess of acidic residues the protein has a net charge of $-8e$[1] or $-9e$, depending on the oxidation state of the metal. The copper atom is embedded 6 Å within the protein and is coordinated by methionine 92, cysteine 84, and histidines 87 and 37. The distorted tetrahedral arrangement of nitrogen and sulfur donors is responsible for the intense blue color of the oxidized protein as well as the high reduction potential, $E°'$[Pc(II)/Pc(I)] = 360 mV at neutral pH (Katoh, 1960). These characteristics are general for higher plant plastocyanins, and the structures of poplar plastocyanin and spinach plastocyanin, which has been used in this study, are almost certainly closely related (Ulrich & Markley, 1978).

The electron-transfer reactions of plastocyanins with cytochrome *f* (Farver & Pecht, 1981; Wood, 1974), flavodoxin (Tollin et al., 1986), azurin (Wood, 1974), and cytochrome *c* (Wood, 1974; Augustin et al., 1983) as well as small molecules such as tris(1,10-phenanthroline)cobalt(III) (Segal & Sykes, 1978; Lappin et al., 1979; Holwerda et al., 1980), ferricyanide (Segal & Sykes, 1978; Lappin et al., 1979), ferrous EDTA (Wherland et al., 1975), flavin (Tollin et al., 1983), flavin semiquinone (Meyer et al., 1987), and ascorbate

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[1] Abbreviations: e, elementary charge in coulombs; dtpa, diethylenetriaminepentaacetate; k_B, Boltzmann's constant; k_0, bimolecular rate constant at zero ionic strength; k_{inf}, bimolecular rate constant at infinite ionic strength; n, number of negative charges; p, number of positive charges; phen, 1,10-phenanthroline; $\bar{r}_P$ and $\bar{r}_N$, radius vectors from the center of mass to the centers of positive and negative charge, respectively; $2A = 1.02$ (eq 5); CDNP, 4-carboxy-2,6-dinitrophenyl; CM, center of mass; Fe(II)*c* and Fe(III)*c*, ferro- and ferricytochrome *c*, respectively; $\mathbf{P}_1$ and $\mathbf{P}_2$, dipole moments of spinach plastocyanin and horse cytochrome *c* in C·m, respectively; Pc(I) and Pc(II), cuprous and cupric plastocyanin, respectively; T, temperature in K; R_1, radius of plastocyanin; R_2, radius of inorganic reactant in eq 3 or cytochrome *c* in eq 4; $R = R_1 + R_2$; Z_i, number of elementary charges; ϵ_0, permittivity constant; ϵ, static dielectric constant; θ, angle between dipole vector and vector from center of mass to site of electron transfer; $\kappa = 0.33\mu^{1/2}A^{-1}$; μ, ionic strength.

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