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Purification to homogeneity and enzymological characterization of a functional covalent complex composed of cytochromes P-450 isozyme 2 and b_5 from rabbit liver

(electron-transfer complex/NADPH-cytochrome P-450 reductase/NADH-cytochrome b_5 reductase/cross-linking/charge pairing)

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ABSTRACT A covalent complex between rabbit hepatic microsomal cytochromes P-450 isozyme 2 (LM_2) and b_5 was created and purified to >95% homogeneity. The purified complex was largely comprised of the two cytochromes covalently attached at the interface of the functional electron transfer–effector complex as shown by the following evidence. (*i*) The spin state of the LM_2 within the complex was greater than the spin state of free LM_2, and the addition of free cytochrome b_5 (cyt b_5) did not further increase the spin state of the LM_2 within the complex. (*ii*) The spectral binding parameters (K_d and ΔA_{max}) for the association of benzphetamine with LM_2 in the complex were identical to those observed with free LM_2 in the presence of saturating concentrations of free cyt b_5 and much different from those observed for LM_2 in the absence of cyt b_5. (*iii*) Reconstituted monooxygenase activity of the covalent LM_2-cyt b_5 complex (LM_2-cyt b_5) in the presence of NADPH-cytochrome P-450 reductase was much higher than the activity of free LM_2 and approached the activity of free LM_2 in the presence of optimal concentrations of free cyt b_5. Furthermore, the K_m for the flavoprotein in supporting either free LM_2 or LM_2-cyt b_5-dependent *p*-nitroanisole demethylation were similar. (*iv*) Less than 20–25% of the cyt b_5 within the complex could be reduced by free NADH-cytochrome b_5 reductase (NADH-cyt b_5 reductase) albeit at a slow rate. The implications of this data to the current understanding of the mechanism and stoichiometry of protein interactions in the hepatic mixed function oxidase system are discussed.

Selective chemical modification of amino and carboxyl groups on several redox proteins has established that many of the protein–protein interactions within electron transport chains are mediated by complementary clusters of amino and carboxyl groups situated in the proximity of the respective redox centers (1–6). Data available for the most extensively investigated systems, in particular those involving cytochrome *c* and/or b_5 (cyt b_5), have generally supported hypothetical models derived from the computer-graphic-assisted manipulation of available x-ray crystal data (7–9).

Water-soluble carbodiimides such as 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC) have been used as zero-distance cross-linking reagents to promote selective amide bond formation between complementary amino and carboxyl groups located on the respective redox proteins at the interface of the electron transfer complex. Using this technique covalent binary complexes between NADH-cyt b_5 reductase and cyt b_5 (10), between cytochrome *c* peroxidase and cytochrome *c* (11), between adrenodoxin and cytochrome P-450_{scc} (12), and between NADPH-cytochrome P-450 reductase and cyt b_5 (13) have been generated. In some cases the covalent complexes have been separated from the unreacted monomers whereupon catalytic (10, 11) studies have often shown the complexes to retain functionality.

Cyt b_5 is well known to effect a stimulation of the activity of reconstituted cytochrome P-450-dependent monooxygenase activity (refs. 14–16, and references cited therein) with certain cytochrome P-450 isozymes. We have shown that the efficacy of these effects was critically dependent on cyt b_5 heme (17) and polypeptide (18) carboxyl groups that were presumed to mediate complex formation with cytochromes P-450 through complementary charge pairing with P-450 cationic groups. We have also shown that low concentrations of EDC will promote the formation of covalent dimers of rat hepatic cyt b_5 and LM_2 specifically through cross-linking of cyt b_5 carboxyl and LM_2 amino groups (19). As a step in the elucidation of the structure of the functional cyt b_5-LM_2 complex, therefore, we now report the successful purification to homogeneity and enzymological characterization of a functional LM_2-cyt b_5 complex generated by EDC-catalyzed cross-linking.

MATERIALS AND METHODS

Purification of Microsomal Enzymes. Rabbit LM_2 and cyt b_5 were purified as described (20, 21). Rat NADPH-cytochrome P-450 reductase and cyt b_5 were purified as described (18), and rat hepatic NADH-cyt b_5 reductase, purified essentially according to ref. 22 was a generous gift from Ingela Jansson. The purified microsomal enzymes exhibited the following specific contents (nmol of holoenzyme per mg of protein): LM_2, 18; rabbit cyt b_5, 45; rat cyt b_5, 42; NADPH-cytochrome P-450 reductase, 11; NADH-cyt b_5 reductase, 35. All enzymes were homogeneous as judged by $NaDodSO_4$/PAGE (23).

Generation and Isolation of the LM_2-cyt b_5 Complex. Cyt b_5 (334 nmol, 1.73 ml) was added to 0.14 ml of a solution of 10% (vol/vol) Emulgen 911 in 50 mM sodium phosphate, pH 7.25/25% (vol/vol) glycerol. LM_2 (334 nmol, 5.6 ml) was then added to this solution with gentle stirring over a 10-min period at room temperature, and the solution remained at room temperature for 1 hr. Benzphetamine·hydrochloride was added to 1 mM final concentration from a 100 mM stock, and after another 1 hr, a solution of EDC (100 mM in H_2O, freshly prepared) was added to attain a final concentration of 9 mM. After EDC addition (2.5 hr), the reaction was terminated by passage of the solution (7.9 ml) through a column of Sephadex G-25 (2.6 × 17 cm) equilibrated with 10 mM sodium phosphate, pH 7.25/20% (vol/vol) glycerol/0.2% Emulgen. Greater than 94% of each starting holocytochrome was

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Abbreviations: LM_2, Rabbit liver microsomal cytochrome P-450 isozyme 2; EDC, 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride; cyt b_5, cytochrome b_5; LM_2-cyt b_5, covalent complex of LM_2 and cyt b_5.

recovered in the column eluate (12.2 ml), which was then applied to a column of DEAE-Sepharose (2.6 × 10 cm) preequilibrated with 10 mM sodium phosphate, pH 7.3/20% (vol/vol) glycerol/0.2% Emulgen (buffer A). Upon loading, an intense red band formed in the top 2–4 mm of the column, but considerable red color was not retained by the column. The column was washed with buffer A until the A_{417} in the eluate decreased to zero whereupon the column was eluted with buffer A in which the ionic strength was increased with sodium phosphate (stepwise) to 25 mM (buffer B), 50 mM (buffer C), 75 mM (buffer D), and 300 mM (buffer E). Elution with each of buffers B–E was discontinued when the A_{417} in the column eluate had decreased to ≤0.015. Fractions eluting with buffers B or C contained substantial amounts of highly pure LM_2-cyt b_5 complex as judged by $NaDodSO_4$/PAGE. The pooled fractions from peaks B and C were dialyzed separately versus 4 liters of 20% (vol/vol) glycerol to attain a conductivity of 0.303 mS at 10°C and were eluted separately through columns of CM-Sepharose (0.9 × 3 cm) equilibrated with 5 mM sodium phosphate, pH 7.3/20% (vol/vol) glycerol. The eluates from these columns contained the LM_2-cyt b_5 complex and were applied directly to columns of hydroxylapatite (0.9 × 4 cm) equilibrated with the last buffer. Once loaded, the columns were washed with 25 mM sodium phosphate, pH 7.6/20% (vol/vol) glycerol/0.2% Emulgen whereupon free cyt b_5 was eluted. Washing was continued with this buffer until free cyt b_5 was no longer detectable in the column eluate. The column was then washed with the above buffer containing no Emulgen until the A_{280} of the eluate decreased to zero. Detergent-free LM_2-cyt b_5 complex was eluted from each column with 300 mM sodium phosphate, pH 7.25, containing 20% (vol/vol) glycerol. The fractions containing the complex were pooled from each column and dialyzed separately in 300 ml of 50 mM sodium phosphate, pH 7.25/25% (vol/vol) glycerol.

Analytical Procedures and Enzyme Assays. All spectra were recorded on a Shimadzu UV-3000 spectrophotometer. Spectral titrations of LM_2 with rabbit liver cyt b_5, rat cyt b_5, or benzphetamine and reconstituted *p*-nitroanisole demethylation were performed at 25°C and analyzed as described (18). Reduction of cyt b_5 by NADH-cyt b_5 reductase was as follows: 0.1 ml of 50 mM sodium phosphate, pH 7.25, containing 25% (vol/vol) glycerol, dilauroyl-α-L-phosphatidylcholine vesicles and NADH-cyt b_5 reductase was incubated for 1 hr then diluted with 0.4 ml of cyt b_5 in the same buffer to attain the following final component concentrations: α-L-dilauroyl-phosphatidylcholine, 627 μM; cyt b_5, 1.54 μM; NADH-cyt b_5 reductase, 0.126 μM. After 3 hr preincubation, the reduction was initiated by the addition of NADH (1 mM final) and monitored at 25°C as the time-dependent increase in $A_{424-437}$. The procedures used for the quantification of cytochrome P-450, and NADPH-cytochrome P-450 reductase have been cited (18). NADH-cyt b_5 reductase was quantified both by its capacity to reduce potassium ferricyanide as described (22) or from the A_{456} of the preparation assuming an absorption coefficient of 10 $mM^{-1} \cdot cm^{-1}$. Cyt b_5 was quantified spectrophotometrically (24) and protein was determined according to Lowry *et al.* (25). Native reconstituted cyt b_5 and cyt b_5 reconstituted with ferric protoporphyrin IX dimethyl ester were prepared from rat hepatic microsomal cyt b_5 as described (17). Mn-cyt b_5 was prepared using the procedure for native reconstituted cyt b_5 synthesis (17), except native heme was replaced with manganese protoporphyrin IX. The final preparation exhibited spectral properties highly similar to those reported for rabbit cyt b_5 reconstituted with manganese protoporphyrin IX (14).

DEAE-Sepharose CL-6B and CM-Sepharose CL-6B were from Pharmacia, EDC and cytochrome *c* were from Sigma, and *p*-nitroanisole was from Eastman. Ferric protoporphyrin IX dimethyl ester was from Porphyrin Products (Logan, UT), and manganese protoporphyrin IX was from Calbiochem.

RESULTS

Reaction of a mixture of LM_2 and cyt b_5 with EDC in a nonionic detergent yielded a small amount (<10% of the total protein) of a higher molecular weight band on subsequent $NaDodSO_4$/PAGE (Fig. 1). The band was not observed following the treatment of LM_2 or cyt b_5 only with EDC (data not shown) and was of the correct size (68 kDa) for a LM_2-cyt b_5 covalent heterodimer. The LM_2-cyt b_5 complex was recovered in highly pure form in the DEAE-Sepharose column eluate after increasing ionic strength of the buffer to 25 mM (peak B). Greater amounts of the complex were recovered in the 50 mM ionic strength eluate (peak C) but both peaks were of indistinguishable purity. Further amounts of the complex were eluted in buffer at 75 mM ionic strength but the fractions were heavily contaminated with unreacted cyt b_5 and were discarded. Fig. 1 shows the $NaDodSO_4$/PAGE analysis of the final preparations of LM_2-cyt b_5 obtained from the further purification of peaks B and C. Both preparations were free of unreacted cyt b_5 and contained <5% contaminating LM_2 monomer. The preparations were assayed spectrophotometrically for their content of holo-cyt b_5 and P-450 and were completely free of cytochrome P-420. Peak B (1 nmol of P-450 total) exhibited a cyt b_5/LM_2 molar ratio of 1.23 and peak C (8.5 nmol of P-450 total) contained more apo-P-450, exhibiting a cyt b_5/LM_2 molar ratio of 1.61. The final preparation of LM_2-cyt b_5 complex purified from DEAE peak C exhibited a specific content (nmol of cyt b_5 heme per mg of Lowry-detectable protein) of 13.1, which is of the order expected for a 68-kDa protein containing 1 mol of holo-cyt b_5 per mol of protein.

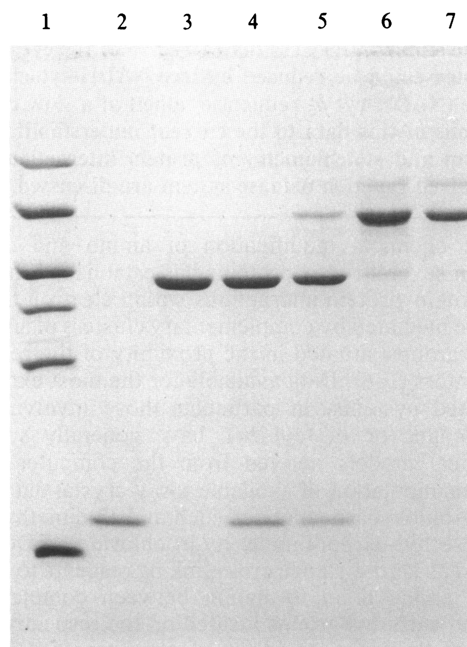

FIG. 1. 10% $NaDodSO_4$/polyacrylamide gel electrophoresis of EDC-catalyzed covalent complexes formed between LM_2 and cyt b_5. Lane 2, cyt b_5 (96 pmol); lane 3, LM_2 (99 pmol); lane 4, LM_2 and cyt b_5 (97 pmol each); lane 5, reaction mixture (96 pmol each of LM_2 and cyt b_5) after EDC; lane 6, LM_2-cyt b_5 complex originating from DEAE peak C (74 pmol of cyt b_5); lane 7, LM_2-cyt b_5 complex originating from DEAE peak B (67 pmol of cyt b_5). Lane 1, molecular size markers (from the *Top*) phosphorylase *a* (92 kDa), bovine serum albumin (67 kDa), glutamate dehydrogenase (53 kDa), creatine phosphokinase (40 kDa), cytochrome *c* (12.5 kDa), and lactate dehydrogenase (36 kDa).

To determine if cyt b_5 was covalently bound to LM_2 to form a functional effector–electron transfer complex, the capacity of LM_2 within the complex both to interact with exogenous substrates and to perform reconstituted substrate oxidation was compared with the corresponding activities of free LM_2 in the absence and presence of free cyt b_5. Benzphetamine interacts with oxidized LM_2 causing a spectrophotometrically detectable conformational change in the hemoprotein. Double reciprocal plots of this change (A_{390}–A_{417}) versus benzphetamine concentration were used to compute the spectral binding parameters (K_d and ΔA_{max}) and are shown in Fig. 2. In the absence of cyt b_5 values of 80 μM and 0.029 were obtained for the substrate dissociation constant and maximal spectral change at infinite saturation, respectively. Preincubation of free LM_2 with either an equimolar or 4-fold molar excess of rabbit liver cyt b_5 increased the LM_2 substrate binding affinity 10-fold as judged by a decrease in K_d to 7–8 μM. Spectral titration of the LM_2-cyt b_5 complex with benzphetamine yielded spectral binding parameters that were very similar to those for titrations of free LM_2 preincubated with saturating concentrations of cyt b_5, and much different from those describing free LM_2–substrate interactions in the absence of free cyt b_5. Excellent linearity of the binding plot for titration of the complex was obtained indicating a uniformity of substrate binding sites within the complex. Under our experimental conditions both rabbit and rat hepatic cyt b_5 bind tightly to free LM_2 causing 24% and 37% increases in high-spin P-450, respectively, at saturation (Table 1). In marked contrast, spectral titration of the LM_2-cyt b_5 complex with rat cyt b_5 failed to cause significant spectrally-detectable conformational changes (Table 1). To examine whether the covalently attached cyt b_5 may be holding the LM_2 in a more high-spin equilibrium configuration within the complex, the Soret spectrum of LM_2 within the complex was analyzed in α-L-dilauroyl-phosphatidylcholine vesicles after carefully offsetting the spectral contribution of the cyt b_5. From the ratio A_{390}/A_{417}, the LM_2 within the complex was calculated to contain about 23% more high-spin cytochrome than free LM_2

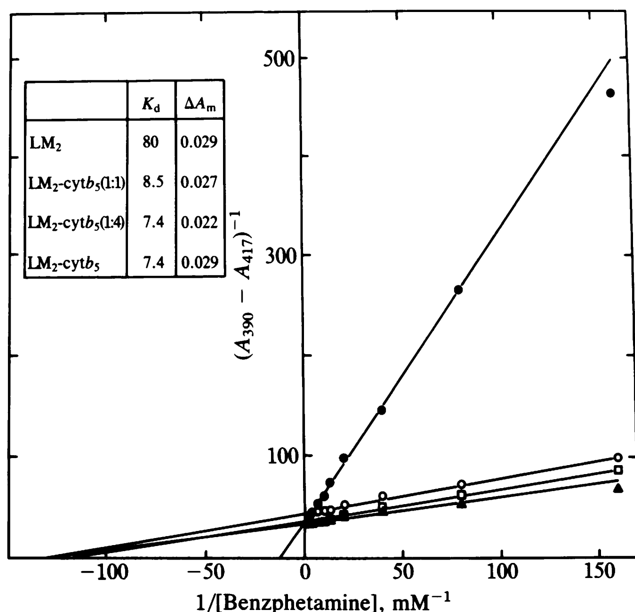

FIG. 2. Effect of covalent complex formation between LM_2 and cyt b_5 on the capacity of LM_2 to interact with benzphetamine. Double reciprocal plots of the spectral change (A_{390} − A_{417}) versus the benzphetamine concentration are shown for the spectral titration of free LM_2 (●), free LM_2 plus equimolar free cyt b_5 (□), free LM_2 plus a 4-fold molar excess of free cyt b_5 (○), and the LM_2-cyt b_5 preparation derived from the DEAE peak C (▲). The *Inset* shows the K_d and ΔA_m for LM_2, LM_2-cyt b_5 molar ratio (1:1), LM_2-cyt b_5 molar ratio (1:4), and LM_2-cyt b_5 complex.

Table 1. The effect of covalent complex formation between LM_2 and cyt b_5 upon the capacity of ferric LM_2 to interact with free cyt b_5

Enzyme	Ligand	K_d, nM	ΔA_{max}	A% high spin	r*
LM_2	Rabbit cyt b_5	20	0.047	37	0.999
LM_2	Rat cyt b_5	25	0.030	23	0.998
LM_2-cyt b_5†	Rat cyt b_5	ND‡	0.001	<2	—

*Correlation coefficient for best fit of Woolf–Hanes plots to biological data.
†The LM_2-cyt b_5 complex used was derived from DEAE peak C.
‡The absorbance changes were too small to quantify accurately and analyze.

Fig. 3*A* compares reconstituted *p*-nitroanisole demethylation catalyzed by the two preparations of LM_2-cyt b_5 complex, free LM_2, or free LM_2 in the presence of free cyt b_5, as a function of P-450-reductase concentration. As reported (18), preincubation of the LM_2-dependent *p*-nitroanisole demethylase system with free cyt b_5 caused a marked stimulation of enzymic activity. The stimulation was greater at lower P-450-reductase concentrations (10-fold) but was nevertheless substantial (4-fold) at higher P-450 reductase levels (Fig. 3*A*). Most importantly, at all P-450 reductase concentrations both preparations of LM_2-cyt b_5 complex displayed demethylation rates that were 4-fold greater than the activity of free LM_2 alone and that approached the rates observed for free LM_2 in the presence of free cyt b_5. The activity of the LM_2-cyt b_5 complex was not affected by the inclusion of free cyt b_5 into the reconstituted system (Table 2). Analysis of

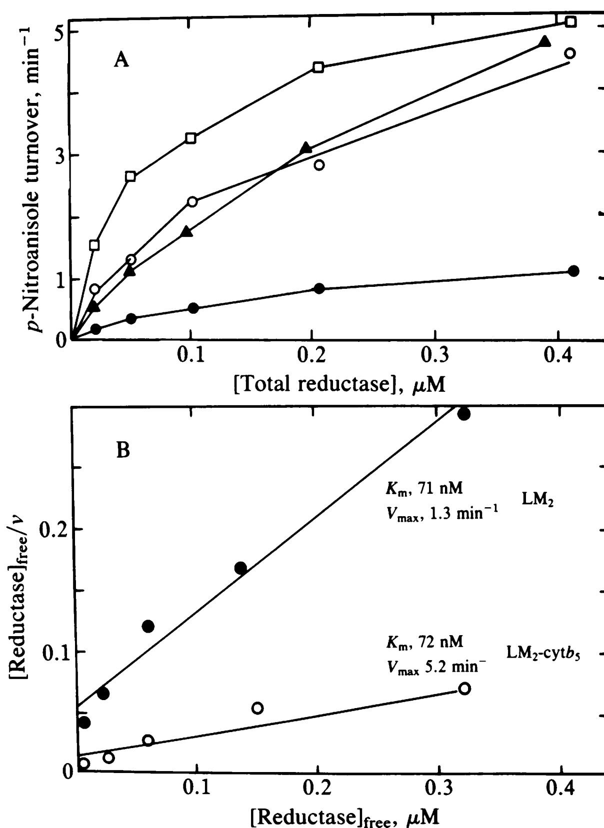

FIG. 3. The effect of covalent complex formation between LM_2 and cyt b_5 on the capacity of LM_2 to catalyze reconstituted *p*-nitroanisole demethylation. (*A*) Variation of reductase concentration (●), free LM_2 plus free equimolar free cyt b_5 (□), LM_2-cyt b_5 complex purified from DEAE peak B (▲), LM_2-cyt b_5 complex purified from DEAE peak C (○). (*B*) Hanes–Woolf plots for the effect of variation in reductase concentration on the *p*-nitroanisole demethylase activity of free LM, (●) and of LM_2-cyt b_5 purified from DEAE peak C (○).

Table 2. Factors influencing the extent of the cyt b_5-mediated stimulation of LM_2-dependent *p*-nitroanisole demethylation

Exp.	Reaction mixture (complete system containing)	*p*-Nitrophenol production, min^{-1}
1	LM_2	0.6
	LM_2/rat cyt b_5 (1:1)	5.1
	LM_2/rat rcyt b_5 (1:1)	5.4
	LM_2/Me_2-cyt b_5 (1:1)	1.8
2	LM_2	0.56
	LM_2/Mn-cyt b_5 (1:1)	0.53
	LM_2/Mn-cyt b_5/rcyt b_5 (1:1:1)	2.4
3	LM_2-cyt b_5	2.7
	LM_2-cyt b_5/rcyt b_5 (1:1)	2.8

Activities were determined at a molar ratio of LM_2/P-450 reductase of 1:1. Other molar ratios are in parentheses. rcyt b_5, Native reconstituted cyt b_5; Me_2-cyt b_5, cyt b_5 reconstituted with ferric protoporphyrin IX dimethyl ester; Mn-cyt b_5, cyt b_5 reconstituted with manganese protoporphyrin IX.

the dependence of demethylase activity on reductase concentration (Fig. 3*B*) showed that at saturating levels of P-450-reductase (V_{max}) the LM_2-cyt b_5 complex was 3 times more active than free LM_2 in *p*-nitroanisole demethylation. However, the apparent K_m for the reductase was completely unaffected by the presence of covalently attached cyt b_5. Thus, although the site of covalent attachment of cyt b_5 to LM_2 within the complex overlaps with the site of interaction with exogenous cyt b_5, this site does not overlap or interfere with the site of functional interaction between LM_2 and P-450-reductase.

In this study we have found cyt b_5 to perform as a redox component in effecting a stimulation of LM_2-dependent *p*-nitroanisole demethylation; manganese protoporphyrin-IX reconstituted cyt b_5 was incapable of stimulating this enzymatic activity (Table 2). The heme propionate charge of native cyt b_5 was also shown to in part mediate the functional interaction with LM_2 as judged by the fact that substitution of native cyt b_5 with cyt b_5 heme dimethyl ester caused only 30% of the stimulation of reconstituted LM_2-dependent substrate metabolism (Table 2).

The extent of electron transfer from NADH-cyt b_5 reductase to cyt b_5 within the LM_2-cyt b_5 complex was examined to determine the accessibility of the cyt b_5. Under the experimental conditions, free cyt b_5 was readily reduced in the presence of free LM_2, reaching 85% of completion by 23 min. In contrast, only about 20% of the total cyt b_5 contained within the covalent complex was reducible, albeit slowly, under the same conditions. When the LM_2-cyt b_5 complex (0.9 μM in cyt b_5) was reconstituted with NADPH-cytochrome P-450 reductase (1 μM), α-L-dilauroyl-phosphatidylcholine (47 μM), benzphetamine (1 mM), and carbon monoxide, the addition of NADPH (0.9 mM) resulted in a concomitant reduction of both cytochromes as judged by subsequent spectrophotometric scanning between 400 and 500 nM. Both cytochromes were reduced to >80% of completion in 23 sec and to completion within 3 min.

DISCUSSION

The present study reports the first successful generation, separation, and characterization of a functional cyt b_5-cytochrome P-450 covalent complex. The two preparations described were shown to possess properties similar to those expected if the two cytochromes were covalently attached at the interface of the functional electron transfer–effector complex. The complex exhibited spectral binding parameters for the type I substrate benzphetamine that were much different from those observed for free LM_2 but very similar to those observed for free LM_2 in the presence of saturating concentrations of free cyt b_5. Further, the spin state of LM_2 within the complex was very similar to the spin state of LM_2 following titration to saturation with rat liver cyt b_5. The increased content of high-spin LM_2 in the complex is probably the reason for the increased substrate binding affinity (26, 27). The complex was much more active than free LM_2 in catalyzing *p*-nitroanisole demethylation regardless of the NADPH-cytochrome P-450 reductase concentration, and the activity of the complex approached that of free LM_2 in the presence of optimal concentrations of free cyt b_5. Approximately 75% of the cyt b_5 in the complex was completely unable to interact with free NADH-cyt b_5 reductase. The 25% that was accessible was reduced only slowly, suggesting that the interaction with this subpopulation was hindered, suboptimal for efficient electron transfer, and probably attached to the LM_2 in a manner that precludes efficient functional interaction. The effector function of cyt b_5 in increasing LM_2 type I substrate binding affinity is apparently less sensitive to the presence of suboptimally bound cyt b_5, as titration of the LM_2-cyt b_5 complex with benzphetamine yielded a single dissociation constant that was much lower than the K_d determined for free LM_2 but like that of free LM_2 in the presence of cyt b_5.

Several important conclusions may be made regarding the mechanism and stoichiometry of protein–protein interactions within the reconstituted system. Firstly, the functional LM_2-cyt b_5 complex is binary. Secondly, since the functional binary complex was selectively generated at low concentrations of EDC, the interface of the functional complex formed between the free proteins most probably contains complementary amino and carboxyl groups situated on the respective proteins in close proximity and in the correct orientation for efficient cross-linking. We showed (18, 19) that the formation of such a covalent complex between rat cyt b_5 and LM_2 exclusively involved participation of LM_2 amino and cyt b_5 carboxyl groups. Since in the present study rabbit liver cyt b_5 was shown to be covalently attached to LM_2 at the same site that recognizes rat liver cyt b_5, and in view of the close structural similarity between rat and rabbit cyt b_5 (28), it is most probable that the covalent LM_2-cyt b_5 complex is similarly cross-linked exclusively through participation of LM_2 amino and cyt b_5 carboxyl groups. Elegant studies have shown the cyt b_5 reductase–cyt b_5 interaction to involve complementary charge pairing of the reductase with a cluster of highly conserved acidic residues located at the exposed heme edge of the cytochrome (3). Accordingly, since about 75% of the cyt b_5 within the LM_2-cyt b_5 complex was completely inaccessible to reduction by free NADH-cyt b_5 reductase, it is most probable that the same acidic site on cyt b_5 is involved in complex formation between cyt b_5 and LM_2. In this scheme the cyt b_5 heme would be in close proximity with LM_2 and accessible for participation in direct electron transfer with LM_2. We demonstrated (17) the involvement of cyt b_5 heme propionate groups in charge pairing with P-450 RLM_5 and a similar involvement of these groups in complex formation with LM_2 is indicated in the present study (Table 2).

Unlike the interactions between cyt b_5 and cyt b_5 reductase, which are diffusion limited, reconstituted P-450-dependent metabolism is saturable at comparatively low P-450 reductase/P-450 molar ratios, and enzymatic activity is proportional to the concentration of the relatively stable reductase-P-450 complex (29). Thus the apparent K_m for the reductase in reconstituted metabolism may be related to the affinity of the two proteins for each other. Accordingly the indistinguishable K_m values for the reductase in supporting the LM_2-cyt b_5 complex or free LM_2-dependent oxidative metabolism shows that covalently attached cyt b_5 does not restrict access of the LM_2 to free NADPH-cytochrome P-450 reductase, and that LM_2 must contain separate binding sites

for the separate interactions with each of these proteins. This may explain why only certain P-450 isozymes (i.e., those possessing a cyt b_5 binding site) can interact with cyt b_5 (14, 15) yet all microsomal P-450 isozymes are capable of interacting with P-450 reductase.

It has generally been assumed that the cyt b_5-mediated stimulation of microsomal or reconstituted P-450-dependent substrate oxidation involves direct reduction of cyt b_5 by either NADH-cyt b_5 reductase or NADPH-cytochrome P-450 reductase (refs. 14–16, 21, and references cited therein). Since both of these reductases apparently interact with the same acidic binding domain on cyt b_5 (3, 4) and since this domain in the covalent complex was largely inaccessible for interaction with free NADH-cyt b_5 reductase, it is likely that the cyt b_5 within the complex is equally inaccessible to direct interaction with NADPH-cytochrome P-450 reductase. Consequently, it is difficult to envision how the increased *p*-nitroanisole demethylase activity of the complex could involve a direct reduction of the cyt b_5 by NADPH-cytochrome P-450 reductase. On the basis of this observation and the demonstration that (*i*) LM_2 possesses separate binding sites for the interactions with cyt b_5 and P-450 reductase, that (*ii*) electron transfer from ferrous P-450-LM_2 to ferric cyt b_5 is facile (18, 30), that (*iii*) the addition of NADPH to a reconstituted system containing the LM_2-cyt b_5 complex and NADPH-cytochrome P-450 reductase results in the reduction of both cytochromes to completion, and that (*iv*) redox reactions involving cyt b_5 are involved in the cyt b_5 stimulation of reconstituted P-450 turnover (refs. 14, 16, and the present study), we propose a reaction pathway to explain the cyt b_5-mediated increase in reconstituted LM_2-dependent substrate oxidation. In this scheme LM_2, cyt b_5, and P-450-reductase form a ternary complex in which the heme prosthetic group of LM_2 is able to participate in redox reactions with either of the other two proteins. First electron input from P-450-reductase bound $FMNH_2$ (E_m, −270 mV, where E_m stands for midpoint potential; ref. 31) to high spin ferric LM_2 (E_m, −180 mV, ref. 32) generates ferrous LM_2 that is then immediately reoxidized to the ferric state by ferric cyt b_5 (E_m, +20 mV, ref. 33). The ferric LM_2 is once again reduced to the ferrous state by the reductase. Subsequent binding of molecular oxygen yields oxyferrous LM_2 (E_m, ca. +50 mV, ref. 34) and triggers electron transfer from ferrous b_5 to oxyferrous LM_2 (26). This scheme is attractive because it does not postulate a requirement for an additional series of protein binding and dissociation reactions in the presence of cyt b_5 and would change the rate of second electron input to a value close to the rate of first electron input from P-450 reductase. This would cause an increase in the rate of oxidation of substrates for which second electron input from P-450 reductase is slower than either first electron input from the reductase or second electron input from ferrous cyt b_5. Also, the decrease in additional binding reactions shortens the delay between first and second electron input possibly causing a decreased likelihood of autoxidation of oxyferrous LM_2. This would support the known capacity of cyt b_5 to increase the net coupling of oxygen activation to product formation (35). Our model also explains why the inclusion of NADH-cyt b_5 reductase (which cannot directly reduce LM_2) into the reconstituted mixed function oxidase system containing cyt b_5 does not significantly increase substrate oxidation (36) and adds significance to the cyt b_5-dependent increase in high-spin ferric LM_2. We have noted (15) that the order in capacity of cyt b_5 to increase the spin state of a series of constitutive P-450 isozymes roughly correlated with the order in capacity of cyt b_5 to stimulate their respective reconstituted turnover with model substrates. In view of the correlations between the rate of P-450 first electron reduction and ferric spin state (37), the above model also predicts that the rate of both first and second electron reduction steps should be increased due to the cyt b_5-dependent increase in high-spin ferric P-450.

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