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Differences in the Mechanism of Functional Interaction between NADPH-Cytochrome P-450 Reductase and Its Redox Partners

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SUMMARY

Selective methylamidation of NADPH-cytochrome P-450 reductase (EC 1.6.2.4) carboxyl groups was used to assess the relative importance of these groups in the enzyme-catalyzed reduction of cytochromes *c*, b_5, and P-450. Methylamidation of as few as 7 mol of carboxyl groups per mol of reductase caused 80% inhibition of cytochrome *c* reduction, 50% inhibition of rat liver microsomal RLM_3 reduction, and up to 90% inhibition in the capacity of the reductase to support reconstituted monooxygenase activities of RLM_3, RLM_5, and LM_2. In marked contrast, cytochrome b_5 reduction measured under comparable conditions was stimulated by 50%. The impaired interactions between the reductase and cytochromes P-450 LM_2 and RLM_5 were shown not to arise from an impaired capacity for the proteins to bind each other but more likely to be due to an inhibition of a step(s) subsequent to complex formation between the oxidized proteins. These results show that the reductase interacts functionally with cytochrome *c* and cytochromes P-450 on the one hand and cytochrome b_5 on the other through different mechanisms.

NADPH-cytochrome P-450 reductase of hepatic endoplasmic reticulum functions in microsomes and reconstituted systems in electron transfer to cytochromes P-450 (1–3), cytochrome b_5 (4, 5), and heme oxygenase (6, 7) as well as fatty acid elongase (8). The mechanism(s) whereby P-450 reductase interacts with these proteins is of considerable interest. An electrostatic mechanism has been implicated for complex formation between the reductase and cytochrome b_5, whereby highly conserved carboxyl groups on b_5 charge pair with complementary cationic groups on the reductase (9). In contrast, NADPH-cytochrome P-450 reductase is also an efficient reductant of the soluble heme protein cytochrome *c*, which forms electron transfer complexes with other redox partners exclusively through electrostatic pairing involving its highly conserved cationic lysyl residues (10–12). Thus, NADPH-cytochrome P-450 reductase may contain separate anionic and cationic binding sites to facilitate interaction with its redox partners (9). Since cytochrome P-450 reductase interacts with cytochrome P-450 as well as with cytochrome b_5, knowledge of the mechanism of interactions between these proteins may contribute to the understanding of the mechanism of the microsomal monooxygenase system.

Interaction between cytochromes P-450 and b_5 in the reconstituted monooxygenase system appears to be through charge pairing between respective amino and carboxyl functional groups (13–15). Assuming both hemoproteins interact with the reductase in the reconstituted monooxygenase system, one might expect them to do so at anionic and cationic sites, respectively, on the reductase. However, in recent studies (13, 14), we observed blocking of protein carboxyl or heme carboxyl groups of cytochrome b_5 to actually facilitate electron transfer between cytochrome b_5 and NADPH-cytochrome P-450 reductase. In view of the ability of this reductase to function in the reconstituted system in the presence of both of these cytochromes (Ref. 16, and references cited therein), it appeared reasonable to examine possible differences in the mechanism of functional interaction between this reductase and redox partners possessing either anionic or cationic residues believed to be involved in their functional interactions. This paper describes, for the first time, studies on chemically modified reductase which demonstrate the presence of two binding domains on NADPH-cytochrome P-450 reductase for its separate interactions with cytochrome b_5 or cytochrome P-450.

Materials and Methods

Purification of microsomal enzymes. Cytochromes P-450 RLM_3 and RLM_5 were isolated from the hepatic microsomes of untreated male CD rats (200 g) according to the method of Cheng and Schenkman (17). LM_2 was purified from the hepatic microsomes of phenobarbital-pretreated male New Zealand rabbits (2 kg) essentially according to the method of Haugen and Coon (18). NADPH-cytochrome P-450 reductase (EC 1.6.2.4) was purified according to the method of Yasukochi and Masters (19) from male CD rats (200 g) pretreated with

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ABBREVIATIONS: RLM, rat liver microsomal cytochrome P-450; LM_2, rabbit liver microsomal cytochrome P-450 isozyme 2; DLPC, L-α-dilauroylphosphatidyl choline; EDC, 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide hydrochloride; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

sodium phenobarbital [0.1% (w/v) in the drinking water for 6 days prior to killing]. Cytochrome b_5 was purified as described previously (20) from the hepatic microsomes of phenobarbital-pretreated rats. All the microsomal proteins used were homogeneous on sodium dodecylsulfate-polyacrylamide gel electrophoresis performed according to the method of Laemmli (21) and were completely free of non-ionic detergents as judged by UV absorbance. The specific contents were as follows (nmol/mg of protein); RLM_3 (16), RLM_5 (12), LM_2 (16), NADPH-cytochrome P-450 reductase (11), cytochrome b_5 (40).

Chemical modification of NADPH-cytochrome P-450 reductase amino groups. The lysyl residues of NADPH-cytochrome P-450 reductase were amidinated as follows. Ethylacetimidate (125 μmol of 1 M stock in 0.5 N sodium bicarbonate, pH 9.5, freshly prepared) was added to 63 nmol of NADPH-cytochrome P-450 reductase (25 μM) in 0.5 N sodium bicarbonate, pH 9.5, containing 20% (v/v) glycerol, and 0.2% (w/v) sodium cholate, at 22°. On hr later a second aliquot of fresh ethylacetimidate was added (125 μmol). A total of six such additions at 1-hr intervals were required to completely amidinate the P-450 reductase. One hr after the last addition, the reaction was terminated by passage of the mixture through a column of Sephadex G-25 (1.3 × 19 cm) previously equilibrated with 50 mM sodium phosphate buffer, pH 7.25, containing 25% (v/v) glycerol. Greater than 80% of the P-450 reductase (fully amidinated) was routinely recovered from the starting protein. The extent of amino group modifications was assessed from the subsequent reactivity of the P-450 reductase with TNBS as described (13).

Acetylation of P-450 reductase lysyl residues was performed as follows. Ten μl of acetic anhydride were added slowly to 2 ml of P-450 reductase (26 μM) in 0.5 N sodium bicarbonate, pH 9.5, 20% glycerol, with gentle stirring at 4°. The reaction was stopped after 2 hr by passage of the mixture through a column of Sephadex G-25 (1.3 × 14 cm) previously equilibrated with 50 mM sodium phosphate, pH 7.25, 25% glycerol. Greater than 90% of the amino groups were modified by this procedure without alteration of the spectral properties of the P-450 reductase.

Chemical modification of P-450 reductase carboxyl groups. Methylamidation of NADPH-cytochrome P-450 reductase carboxyl groups was performed using ^{14}C-methylamine in the presence of EDC. To preclude EDC-catalyzed cross-linking of proteins, the amino groups on the reductase were blocked by prior amidination. Methylamidination was performed as follows: solid methylamine hydrochloride and ^{14}C-methylamine hydrochloride (46 mCi/mmol), were added to the amidinated P-450 reductase (45 μM) in 50 mM sodium phosphate, pH 7.25, 25% glycerol to attain a concentration of 0.18 M, and specific activity of 280–470 cpm nmol^{-1}. Twelve nmol of the medium were applied to a column of Sephadex G-25 (1 × 11 cm) equilibrated with 50 mM sodium phosphate, pH 7.25, 25% glycerol, and the radioactivity recovered in the eluted protein was used to correct subsequent reaction time points for nonspecific ^{14}C-methylamine binding. The amount of nonspecific binding was less than 15% of the radioactivity recovered at the earliest time points. The methylamidation reaction was then initiated by the addition of solid EDC to 0.18 M final concentration. At various times, 10-nmol aliquots of P-450 reductase were removed and applied to Sephadex G-25 as described above for the pre-zero time point. The extent of modification of the P-450 reductase was calculated from the ratio A_{456nm} of P-450 reductase/cpm ml^{-1} after correction for nonspecific binding, and using the experimentally determined specific activity of ^{14}C-methylamine in the reaction mixture.

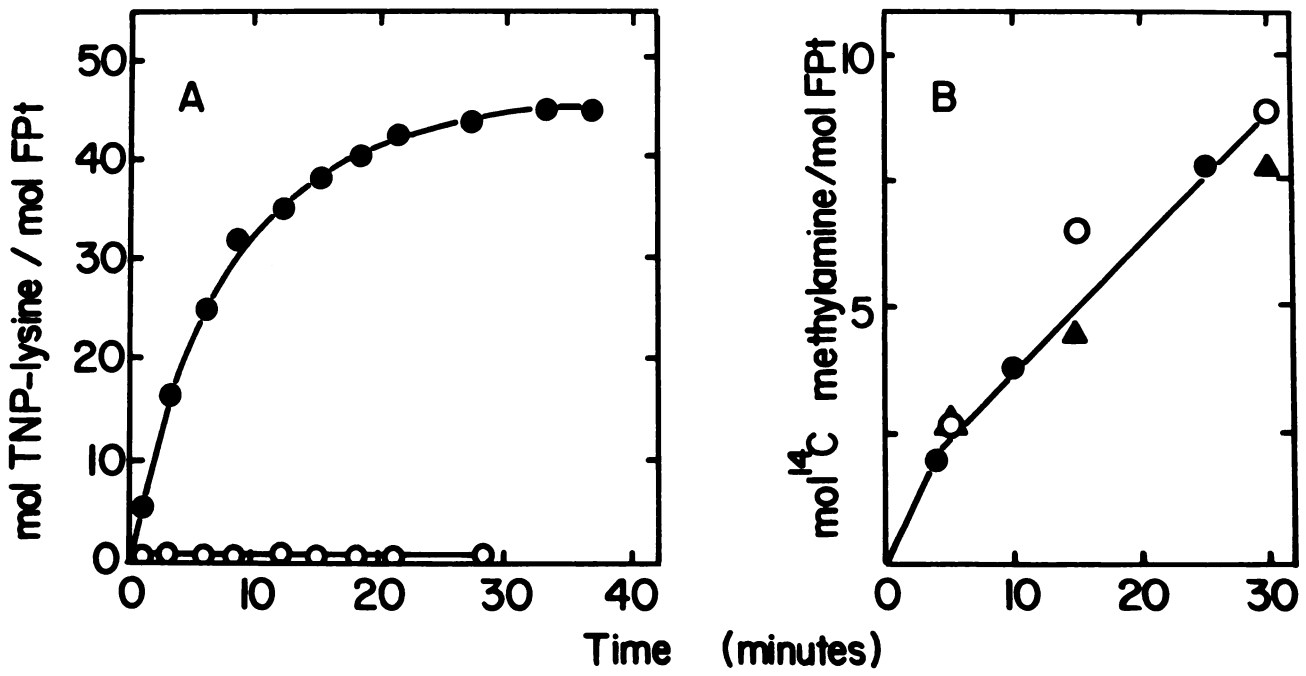
Analytical procedures and enzyme assays. All spectra were recorded on a Shimadzu UV-3000 spectrophotometer. Cytochromes P-450 were quantified according to the method of Omura and Sato (22) using a value of 91 mM^{-1} cm^{-1} for the difference absorption coefficient of carbonmonoxyferrous versus ferrous cytochrome. Cytochrome b_5 was quantified using a value of 112 mM cm^{-1} for the ferric/ferrous difference absorption coefficient (23) between 424 and 490 nm, and NADPH-cytochrome P-450 reductase was quantified using a value of 21.4 mM^{-1} cm^{-1} at 456 nm for the oxidized P-450 reductase (24).

Spectral complex formation between the P-450 reductase and RLM_5 was performed and analyzed as described previously for spectral complex formation between cytochrome b_5 and RLM_5 (13). Cytochrome P-450-dependent substrate metabolism was assayed at 25° in a reconstituted system containing cytochrome P-450 (0.15–0.18 μM), DLPC vesicles (32 μM), and the indicated amounts of P-450 reductase. Prior to dilution with 50 mM sodium phosphate buffer, pH 7.25, 25% glycerol, and substrates, the stock proteins and lipids were preincubated for 30 min. Reactions were initiated via the addition of NADPH (0.5 mM final concentration). The *O*-deethylation of 7-ethoxy coumarin (1 mM) was assayed according to the method of Ullrich and Weber (25). Benzphetamine (1 mM) demethylation was followed as the release of formaldehyde, and was detected according to the method of Nash (26). Testosterone hydroxylation (0.12 mM) was assayed as previously described (17).

Electron transfer from the reductase to RLM_3 or LM_2 was monitored at room temperature using a Dionex stopped-flow spectrophotometer as follows. Cytochrome P-450 and NADPH-cytochrome P-450 reductase stock solutions were preincubated at 22° for 30 min with DLPC vesicles (stock 5 mg ml^{-1}) prepared according to the method of Jansson *et al.* (16). The solution was diluted with 50 mM sodium phosphate buffer, pH 7.25, 25% glycerol containing glucose (7.4 mM), and either benzphetamine or testosterone, and bubbled with carbon monoxide for 5 min. A stock solution of glucose oxidase (18 units ml^{-1}) and catalase (590 units ml^{-1}) was added, and the solution was loaded into an air-tight drive syringe of the stopped-flow instrument. An equal volume of buffer containing glucose (7.4) and NADPH (0.8 mM) was similarly treated and loaded into the second drive syringe. Under these conditions anaerobiosis is achieved within about 3 min as judged using an oxygen electrode. After at least 3 min preequilibration, reactions were initiated by rapidly mixing equal volumes (0.25 ml) of the two solutions, and the time course of reduction was followed as the formation of carbonmonoxyferrous P-450 at 450 nm. Data were collected using a Horizon II Northstar microcomputer in conjunction with the stopped-flow single-channel data collection programme (version 5.02, On Line Instruments, Jefferson, GA). The following final concentrations of components of the reconstituted system after mixing were: for RLM_3 reduction, RLM_3 (0.5 μM), P-450 reductase (0.5 μM), DLPC (105 μM), testosterone (120 μM); for LM_2 reduction, LM_2 (0.25 μM), reductase (0.25 μM), DLPC (52 μM), benzphetamine (1 mM). Ethylacetimidate, EDC, methylamine hydrochloride, TNBS, and cytochrome *c* (horse heart) were from Sigma, 1-^{14}C-Methylamine was from New England Nuclear and 7-ethoxycoumarin was from Aldrich.

Results

Treatment of NADPH-cytochrome P-450 reductase with ethylacetimidate, as described in Materials and Methods, resulted in full amidination of the enzyme as demonstrated (Fig. 1A) by its subsequent lack of reactivity with TNBS. Absorption of the reductase at its UV peak (275 nm) and its near UV/visible peaks (380 nm, 456 nm) was monitored to assure no loss of flavin mononucleotide or structural integrity. The fully amidinated protein in the oxidized state had the same extinction ratios at 275 nm/456 nm and 275 nm/280 nm as the native reductase (Table 1) and, further, was 50% more active in the reduction of potassium ferricyanide. Amidination caused the formation of covalent dimers of the reductase which did not separate from the monomeric reductase on Sephacryl S-200. Such intermolecular cross-linking has been shown to occur under certain conditions during amidination of other proteins, and the side reactions responsible for the intermolecular cross-linking have been described (27). Subsequent amidinations were performed in the presence of 0.2% sodium cholate. This served to disperse the protein aggregates and minimized covalent cross-linking to less than 10% of the total reductase (Fig.



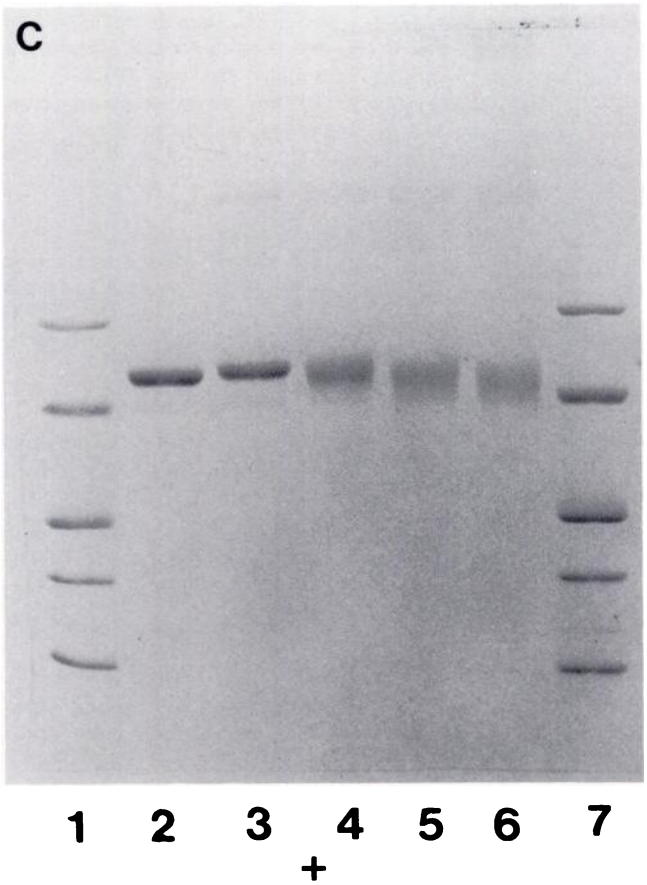


Fig. 1. Chemical modification of NADPH-cytochrome P-450 reductase. A. The effect of amidination of P-450 reductase upon the extent of its subsequent reactivity with TNBS: ○, amidinated; ●, unmodified. B. Time course of methylamidation of P-450 reductase carboxyl groups. Data for three separate experiments are shown. C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of chemically modified P-450 reductase. Forty pmol each of amidinated P-450 reductase containing 0 (*track 3*), 2.7 (*track 4*), 6.6 (*track 5*), and 9 (*track 6*) mol of ^{14}C-methylamine/mol of P-450 reductase. *Track 2* contains unmodified P-450 reductase and *tracks 1* and *7* contain molecular weight marker proteins: phosphorylase *a*, 92,500; bovine serum albumin, 68,000; glutamic dehydrogenase, 53,000; ovalbumin, 43,000; lactic dehydrogenase, 36,000. All Coomassie blue-detectable protein entered the separating gel. Migration was from top to bottom.

TABLE 1

The effect of chemical modification of NADPH-cytochrome P-450 reductase upon the functional integrity of the reductase[a]

Modification	mol ^{14}C-Methylamine/mol reductase	A_{275}/A_{456}	A_{275}/A_{580}	Ferricyanide reductase activity
				min^{-1}
Unmodified	0	8.7	10.1	1819
Amidinated	0	8.7	10.1	2908
Amidinated	2.7	8.8	10.1	2908
Amidinated	6.6	8.9	10	3344
Amidinated	9.0	9.0	9.9	3126

[a] The reductase was fully amidinated prior to methylamidation to circumvent protein cross-linking.

1C). The presence of glycerol to stabilize the reductase precluded the use of sodium borate buffers (9), since borates form acidic chelates with glycerol (28). Thus, a bicarbonate buffer was used instead. The fully amidinated reductase when reacted with ^{14}C-methylamine and a water-soluble carbodiimide resulted in a time-dependent methylamidation of its carboxyl groups (Fig. 1B) without proteolysis or cross-linking of reductase molecules (Fig. 1C). By 30 min, up to 9 mol of carboxyl groups per mol of reductase were methylamidated (Fig. 1B). Rat liver NADPH-cytochrome P-450 reductase contains a total of 144 side chain carboxyl groups per mol of the reductase, some of which are in the form of asparagine and glutamine (24). Treatment of the modified protein with 0.5 M hydroxylamine failed to liberate radiolabel, suggesting that no significant modification of tyrosyl residues had occurred during the reaction (29). As seen in Table 1, methylamidation of the amidinated reductase altered neither its spectral characteristics nor its capacity to reduce ferricyanide.

In view of the enhanced turnover of the amidinated enzyme observed with ferricyanide (Table 1), the influence of amidination on electron transfer to other redox partners was examined (Table 2). Complete amidination of the reductase differentially influences its capacity to transfer electrons to different acceptors. Thus, the initial rates of cytochrome *c* and cytochrome b_5 reduction were stimulated 1.15-fold and 2.5-fold, respectively, by amidination. In contrast, the fast phase rates of RLM_3 and LM_2 reduction by the amidinated reductase were inhibited 60 and 40%, respectively (Table 2). Although input of the first electron to cytochrome P-450 was inhibited by amidination, oxidation of different substrates by the different cytochromes P-450 was stimulated by as little as 38% to as much as 800% (Table 2).

Figure 2 demonstrates the effect of carboxyl group modification of fully amidinated NADPH-cytochrome P-450 reductase on its ability to transfer electrons to cytochrome *c* as a function of the number of residues modified. A strong inhibition of reduction was obtained, with loss of more than 93% of turnover after modification of as few as 9 mol of carboxyl groups/mol of enzyme. In contrast to the inhibition seen in cytochrome *c* turnover, methylamidation of 6.6 mol of carboxyls/mol of reductase enhanced the turnover of cytochrome b_5 by about 50% (Fig. 3). Further modification of up to 9 mol of carboxyl residues/mol of reductase attenuated the stimulation (Fig. 3). Despite these effects, the reductase retained its capacity to bind NADPH and accept electrons, since ferricyanide reductase activity was unaffected by methylamidation (Table 1).

The influence of methylamidation of NADPH-cytochrome P-450 reductase on first electron transfer to ferric cytochrome P-450 RLM_3 was examined. As discussed elsewhere (30), the reduction can be separated for simplicity into two sequential pseudo-first order phases. The fast phase of reduction was most sensitive to carboxyl group modification (Fig. 4), with about a 60% decline in the apparent fast phase rate constant at 9 mol modified per mol of amidinated reductase. No effect was seen on the apparent rate constant from the slow phase (0.0012 S^{-1}). Similar effects upon LM_2 fast phase reduction were observed, with the rate constant decreasing from 0.43 ± 0.01 S^{-1} to 0.26 ± 0.03 S^{-1} after modification of 9 mol of carboxyl groups/mol amidinated reductase.

Compared with its effect on first electron reduction of RLM_3, methylamidation of the amidinated reductase caused a strong inhibition of the turnover of this enzyme with 7-ethoxycoumarin as substrate (Fig. 5). Eighty per cent inhibition occurred at 2.7 mol of the carboxyl group modified per mol of enzyme versus only 18% inhibition of the fast phase of one electron

TABLE 2

The effect of complete amidination of NADPH-cytochrome P-450 reductase upon its capacity for functional interaction with various electron acceptors

Reaction	Turnover[b]	
	Unmodified	Amidinated
	min^{-1}	
Cytochrome *c* reduction	1676 ± 95	1928 ± 111
Cytochrome b_5 reduction	54	126
Cytochrome P-450 LM_2 reduction	63.9 ± 2.8	25.8 ± 0.7
Cytochrome P-450 RLM_3 reduction	15.6 ± 0.1	9.9 ± 0.2
LM_2-dependent benzphetamine demethylation[a]	13 ± 2	18 ± 1
LM_2-dependent 7-ethoxycoumarin *O*-deethylation[a]	0.57	4.61
RLM_5-dependent 7-ethoxycoumarin *O*-deethylation[a]	0.35	0.85
RLM_3-dependent 7-ethoxycoumarin *O*-deethylation[a]	0.18	0.38
RLM3-dependent testosterone 6β-hydroxylation[a]	0.28	0.42

[a] Activities were determined at a 1:1 molar ratio of P-450 to reductase.
[b] Turnover values were extrapolated to $t = 0$.

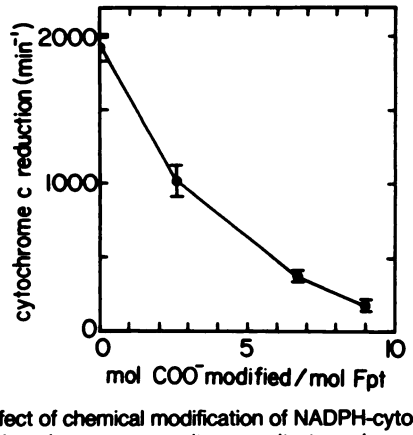


Fig. 2. The effect of chemical modification of NADPH-cytochrome P-450 reductase carboxyl groups upon its capacity to reduce cytochrome *c*. The reaction was measured in a 3-ml volume comprising cytochrome *c* (72 μM) and the reductase (0.015 μM) in 50 mM sodium phosphate buffer, pH 7.25, 25% glycerol, at 22°C. The reaction was initiated by the addition of NADPH (0.4 mM final) via a plunger and monitored as an increase in $\Delta A_{(550-541)nm}$. *Ordinate* values are turnover numbers, extrapolated to $t = 0$.

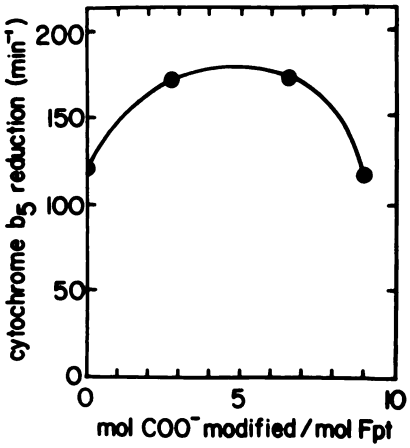


Fig. 3. The effect of chemical modification of NADPH-cytochrome P-450 reductase carboxyl groups upon NADPH-cytochrome b_5 reductase activity. The reaction was measured in a 3-ml volume containing cytochrome b_5 (3 μM), the reductase (0.1 μM), and DLPC (150 μg ml^{-1}), in 50 mM sodium phosphate, pH 7.25, 25% glycerol, at 22°. Prior to dilution, cytochrome b_5, P-450 reductase, and lipid were preincubated at room temperature for 2.5 hr. The 3-ml system was bubbled with nitrogen for 5 min prior to initiation of the reaction with NADPH (0.4 mM final). The time course of reduction was followed as an increase in $\Delta A_{(424-437nm)}$. *Ordinate* values are turnover numbers extrapolated to $t = 0$.

reduction of the ferric RLM_3 (Fig. 4). This monooxygenase exhibited the slowest turnover of 7-ethoxycoumarin of the cytochromes P-450 tested (Table 2). The more active RLM_5 and the much more active LM_2 were less sensitive to inhibition of 7-ethoxycoumarin deethylase activities by methylamidation (Fig. 5) but, nevertheless, had less than 10% of control activity remaining with 9 mol of carboxyl groups modified per mol of reductase. Despite the fact that the amidinated reductase supported rates of LM_2-dependent benzphetamine turnover about 5 times faster than LM_2-dependent 7-ethoxycoumarin turnover, both activities were equally sensitive to inhibition following methylamidation of the reductase. Methylamidation of the reductase also strongly inhibited RLM_3-dependent 6β-hydroxylation of testosterone (Fig. 5).

In view of the precedent set for the involvement of electrostatic mechanisms of complex formation between redox proteins, studies were performed to examine the requirements for carboxyl and amino residues of NADPH-cytochrome P-450 reductase in functional complex formation with cytochrome P-450. The effects of variation of reductase concentration on LM_2-catalyzed 7-ethoxycoumarin *O*-deethylation are shown in Table 3. Amidination decreased the K_m values[1] for the reductase in metabolism by a factor of 4.5 while increasing the V_{max} by a factor of 3.1. Subsequent methylamidation of as few as 7.2 mol of carboxyl residues/mol of amidinated reductase had little effect on the lowered K_m for reductase and decreased V_{max} to a value similar to that of the unmodified reductase. The lack of effect on K_m for the reductase with strong inhibitory effects on V_{max} suggests that carboxyl group modification does not influence the affinity of the reductase for cytochrome P-450, but does influence the functionality of the complex. Since the reductase is capable of perturbing the Soret absorption of RLM_5, this was used as a means to quantify the binding between reductase and cytochrome P-450 (Table 4). The dissociation constant of RLM_5 for unmodified NADPH-cytochrome P-450 reductase was 0.213 μM, indicating fairly tight binding. Acetylation of 90% of the reductase amino groups, which removes cationic charges on the reductase amino groups, as well as full amidination, was without significant influence on either the dissociation constant for the complex or the magnitude of the maximal spectral change (ΔA_{max}). Even methylamidation of up to 7.2 mol of carboxyl residues/mol of reductase affected neither the K_d nor the A_{max} for spectral complex formation (Table 4).

Discussion

In this study the mechanism of electron transfer and interaction between NADPH-cytochrome P-450 reductase and a number of hemoproteins has been examined through the use of chemical modification of the reductase. The studies used either fully amidinated reductase or 90% acetylated reductase or fully amidinated reductase with varying extents of carboxyl residue modification. No more than 9 mol of carboxyl residues/mol of reductase were modified.

Amidination of the P-450 reductase was performed as a precaution to prevent carbodiimide-dependent protein cross-linking during methylamidation of the carboxyl groups. However, some interesting effects upon enzymic activity were noted following amidination. For example amidination stimulated P-450 reductase-dependent ferricyanide and cytochrome b_5 reduction as well as the capacity of the P-450 reductase to support reconstituted P-450-dependent mixed function oxidase activity. The amidinated P-450 reductase was less active in cytochrome P-450 reduction, however. In view of the large number of free amino groups modified by this treatment with retention of net charge, it is not possible to interpret these effects in terms of alterations in specific protein structural features or binding sites. However, the substantial activity of the amidinated P-450 reductase with all of the acceptors tested made it a useful enzyme derivative with which to study the effect of subsequent carboxyl group methylamidation upon enzymic activity.

The P-450 reductase carboxyl groups most accessible to methylamidation were shown to be essential for efficient elec-

[1] Wherever K_m values are given, the meaning is "apparent" K_m since we are dealing with membrane enzymes and vesicles, where enzymes must partition between the aqueous solution and vesicles.

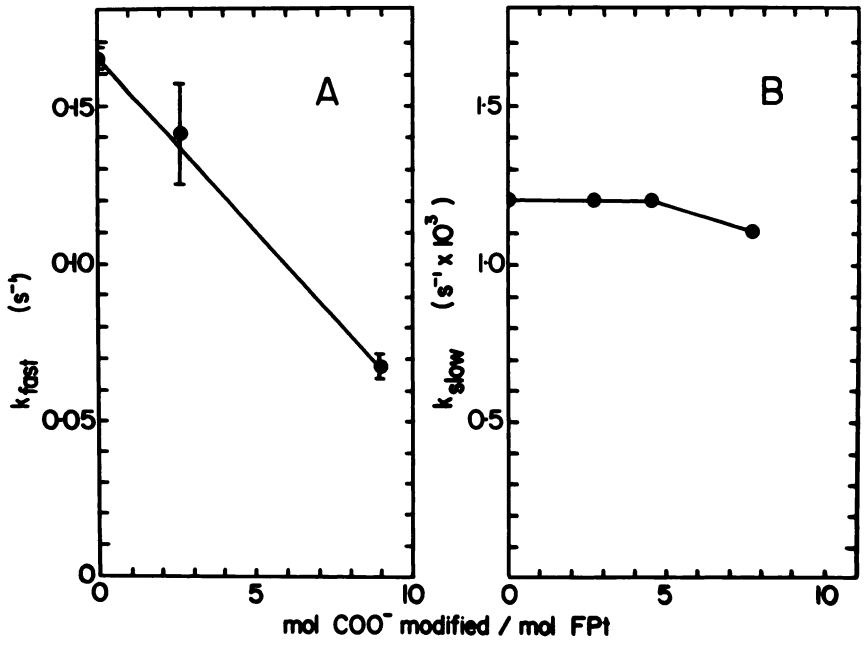


Fig. 4. The effect of chemical modification of NADPH-cytochrome P-450 reductase upon its capacity to reduce cytochrome P-450 RLM3. A. Fast phase rate constants determined in the first 2 sec of the reaction; B. slow phase rate constants determined between 3 and 25 min after initiation of the reaction.

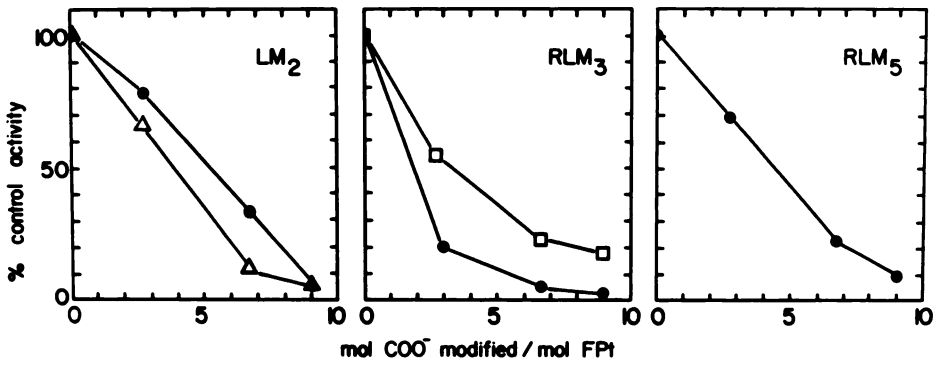


Fig. 5. The effect of chemical modification of NADPH-cytochrome P-450 reductase carboxyl groups upon the capacity of the P-450 reductase to support reconstituted cytochrome P-450-dependent monooxygenase activity. ●, activities with 7-ethoxycoumarin as substrate; △, activities with benzphetamine as substrate; □, 6β-hydroxylase activity with testosterone as substrate. Activities were determined at equimolar cytochrome P-450 and reductase and are expressed as percentage of the activities in the presence of amidinated P-450 reductase given in Table 2.

TABLE 3

Kinetic parameters for the variation of NADPH-cytochrome P-450 reductase concentration during LM_2-catalyzed 7-ethoxycoumarin O-deethylation: effect of chemical modification of the reductase

Modification	Kinetic parameters[a]	
	K_m	V_{max}
	nM	min^{-1}
Unmodified	378	2.9
Amidinated	84	9.1
Amidinated (contains 7.2 mol ^{14}C-methylamine mol^{-1} reductase)	108	3.4

[a] Determined over the range in molar ratio (P-450 reductase/P-450) of 0.25:9. Data were analyzed using an iterative Hanes-Woolf analysis (13).

TABLE 4

The effect of chemical modification of NADPH-cytochrome P-450 reductase upon its capacity for spectral complex formation with RLM_5

Modification	mol ^{14}C-Methylamine/mol reductase	K_d[a]	$\Delta\epsilon_{max}$[a]
		nM	$mM^{-1}\ cm^{-1}$
Unmodified	0	213 (52)[b]	47
Acetylated	0	288 (71)	54
Amidinated	0	350 (80)	38
Amidinated/methylamidated	7.2	329 (103)	34

[a] Spectral binding parameters were determined using an iterative Hanes-Woolf analysis (13). Differences in absorption between 420 nm and 390 nm were recorded.
[b] Numbers in parentheses, standard deviations of the K_d.

tron transfer both to cytochrome *c* and to the cytochromes P-450, as well as in supporting reconstituted cytochrome P-450-dependent mixed function oxidase activity. Inhibition of these enzymic activities was not due to an impairment of NADPH binding and activation, since ferricyanide reductase activity was essentially unchanged by progressive methylamidation. Rather, since the number of carboxyl groups modified was very small in relation to the total number of acidic residues in P-450 reductase, the effects of chemical modification upon functional interaction with its redox partners are best explained in terms of altrations in electrostatic mechanisms of complex formation between the reductase and the above hemoproteins, involving NADPH-cytochrome P-450 reductase carboxyl group charge.

The quantitative differences in the extent of inhibition seen by methylamidation of P-450 reductase carboxyl groups on one electron transfer to cytochrome *c* (2.5 residues = I_{50}) or cytochrome P-450 (8 residues = I_{50} under CO) may reflect differences in the mechanism of complex formation in each case. P-450 reductase has a hydrophilic catalytic segment and a hydrophobic membrane binding segment which serves to bind the enzyme to the membrane (31). It is known that electron transfer to cytochromes P-450 requires the amphipathic form of the P-

450 reductase, whereas electron transfer to cytochrome *c* does not (24).

Neutralization of P-450 reductase carboxyl groups dramatically impaired reconstituted P-450-dependent substrate turnover. This effect was seen as a decrease in V_{max} without an effect on K_m for the reductase. Furthermore, methylamidation of the reductase did not alter the K_d for spectral complex formation between the reductase and cytochrome P-450 (Table 3). The data suggest a role for P-450 reductase carboxyl groups at some step subsequent to complex formation in substrate monooxygenation. It is conceivable that complex formation is primarily driven through hydrophobic interactions involving the P-450 reductase membrane-binding segment which provide the correct proximity and orientation of the proteins for a secondary electrostatic interaction at the locus of the redox centers. In this schema, the electrostatic component would be mediated through P-450 reductase carboxyl groups and complementary charged residues on P-450 and confers functionality.

The above model could also explain the quantitative differences in the effects of P-450 reductase carboxyl group modification on cytochrome *c* and cytochrome P-450 reduction. In the case of cytochrome *c* reduction, inhibition of charge pairing between the two proteins would completely inhibit facilitated complex formation with the residual electron transfer rate resulting solely from three-dimensional random collision of the protein components in the aqueous solution. Conversely, since cytochrome P-450 and NADPH-cytochrome P-450 reductase are anchored to the lipid bilayer through their hydrophobic segments, inhibition of charge pairing would still enable the proteins to interact through two-dimensional random collision. In addition, a direct interaction between the hydrophobic segments on the two proteins within the phospholipid bilayer might hold the molecules close enough together to enhance the frequency of random collisions of the respective redox centers.

The reason for the quantitative differences in the effect of P-450 reductase carboxyl residue neutralization on P-450 first electron reduction and substrate oxidation may involve the requirement for a second electron for the latter process. It is possible that inhibition of both steps causes more severe effects to be seen on substrate turnover. Alternatively, the second electron input step may be more sensitive to the modification of the reductase. At present these are possibilities that cannot be directly tested.

Fast phase reduction of cytochrome P-450 involves the high spin ferric form of P-450 (30) and is probably due to electron transfer within preformed complexes of P-450 reductase and P-450 (32). The 60% attenuation of RLM_3 fast phase reduction following modification of about 9 mol of carboxyl groups/mol of P-450 reductase may, therefore, be due to inhibition of electron transfer within the complexes. Since metabolism and only fast phase reduction of RLM_3 were inhibited by P-450 reductase carboxyl modification, it is concluded that only fast phase reduction is functional in metabolism.

Electron transfer between NADPH-P-450 reductase and both cytochromes P-450 and b_5 is important in the cytochrome b_5-mediated stimulation of turnover of some forms of P-450 with some substrates (33, 34).[2] The reduction of cytochrome b_5 involves cytochrome b_5 carboxyl residues and a presumed complementary cationic site on NADPH-P-450 reductase. Since the data provided here indicated the involvement of anionic residues on NADPH-P-450 reductase in mediating functional interactions with cytochromes P-450 and *c*, this raises the possibility of different mechanisms and possibly discrete binding sites on the reductase to facilitate interaction with either cytochromes P-450 and *c*, on the one hand, or with cytochrome b_5, on the other. This hypothesis was tested by examining the capacity of carboxy-modified NADPH-cytochrome P-450 reductase to reduce cytochrome b_5. In sharp contrast to the effects on cytochrome *c* or P-450 reduction, b_5 reduction was not inhibited but was actually *stimulated* by methylamidation of up to 6.6 mol of carboxyl/mol of P-450 reductase. Methylamidation of 9 mol of carboxyl/mol of P-450 reductase caused some loss of the stimulation. It is, therefore, concluded that the P-450 reductase carboxyl residues most reactive in carbodiimide-catalyzed methylamidation and essential in cytochrome *c* reduction are not involved in cytochrome b_5 reduction. This raises the possibility of separate sites in mediating interactions with cytochrome *c* or P-450, on the one hand, and cytochrome b_5, on the other. Furthermore, the observed *stimulation* of cytochrome b_5 reduction by methylamidation of the reductase may be due to neutralization of charge repulsion between the carboxyl residues on the reductase and carboxyl residues on cytochrome b_5.

Previous studies from this laboratory (13–15) have provided evidence for the involvement of electrostatic mechanisms in complex formation between components of the hepatic microsomal P-450 monooxygenase system. These components include cytochromes P-450, b_5, and NADPH-cytochrome P-450 reductase. Protein carboxyl residues and heme propionic acid residues of cytochrome b_5 were shown to be necessary for functional interaction of this protein with P-450 but inhibitory of functional interaction with P-450 reductase. Whereas the functional competence of the P-450 reductase-P-450 complex was shown to be dependent upon P-450 reductase carboxyl groups, that of the P-450 reductase-b_5 complex was not, suggesting that interaction of NADPH-cytochrome P-450 reductase with P-450 involves binding of the hemoprotein to a site different from that involved in cytochrome b_5 interaction with P-450 reductase. The effects of methylamidation of NADPH-cytochrome P-450 reductase, or cytochrome b_5 (13), on the spectral interaction of these proteins with RLM_5 differed. The interaction with cytochrome b_5 was strongly inhibited, whereas the interaction of the reductase was hardly inhibited, suggesting differences in the mechanism of interaction of these proteins with RLM_5.

Our conclusions from these studies are consistent with the data of other laboratories. For example, chemical modification of cytochrome P-450 lysyl groups (35, 36) inhibited functional interactions with P-450 reductase, suggesting an electrostatic mechanism of complex formation between these groups and anionic residues on the reductase. Furthermore, cytochrome *c*, which is also reduced by P-450 reductase, has been shown to interact with several proteins through participation of a cluster of highly conserved lysyl residues which charge pair with complementary residues on the redox partner (10–12). Finally, cytochrome b_5-P-450 reductase interactions involve b_5 carboxyl groups which presumably charge pair with cationic groups on the reductase (9).

[2] P. P. Tamburini and J. B. Schenkman, unpublished data.

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