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THE NH_2-TERMINAL REGION OF RABBIT CYP2E1 IS NOT ESSENTIAL FOR INTERACTION WITH NADPH-CYTOCHROME P450 REDUCTASE

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Recently we reported that electrostatic forces interfere with the formation of the NADPH-cytochrome P450 reductase/cytochrome P450 electron transfer complex and suggested that this complex is formed by the attraction of the complementary hydrophobic patches (Voznesensky, A., and Schenkman, J. (1994) **269** J. Biol. Chem, 15724-1573). In this report we evaluate the role of the NH_2-terminal hydrophobic region of CYP2E1 in the interaction with the reductase by comparing ionic strength dependence of the reduction of the full-length and truncated Δ3-29 CYP2E1. Increasing ionic strength stimulates reduction of both full-length and truncated CYP2E1. The neutralization of electrostatic interactions by increasing ionic strength revealed no impairment of the Δ3-29 CYP2E1 reduction compared to that of the full-length CYP2E1, indicating that the NH_2-terminal region is not essential for the interaction of the cytochrome with the reductase.

Microsomal cytochrome P450 is a family of integral membrane proteins responsible for the metabolism of numerous endogenous and exogenous compounds. Electrons needed for these metabolic transformations are supplied by NADPH via another integral membrane protein, NADPH-cytochrome P450 reductase. Electrons are transferred from the reductase to the cytochrome P450 within the protein-protein complex. Recently we presented evidence that formation of this complex between the reductase and several cytochrome P450 enzymes is inhibited by electrostatic repulsion (1-3). We suggested that the electron transfer complex is formed by attraction of the complimentary hydrophobic patches on the cytochrome and the reductase (1). The hydrophobicity of the amino-terminus is highly conserved in the cytochrome P450 superfamily and this region is a potential candidate for the site of the hydrophobic interaction with the reductase. In the present study we investigated the role of the amino-terminal region of CYP2E1 by comparing reduction of the full-length CYP2E1 and truncated Δ3-29 CYP2E1 at varying ionic strength. Increasing ionic strength gradually neutralizes electrostatic interactions, making it a useful tool for the study of NADPH-cytochrome P450 reductase/cytochrome P450 interactions. Deletion of amino acids 3-29 not only did not inhibit

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cytochrome P450 reduction but actually enhanced reducibility of the cytochrome, suggesting that the terminal hydrophobic region is not essential for the interaction of CYP2E1 with the NADPH-cytochrome P450 reductase.

MATERIALS AND METHODS

Materials. 4-Methylpyrazole, reduced β-nicotinamide adenine dinucleotide phosphate, D(+)glucose, glucose oxidase, and catalase were obtained from Sigma Chemical Co. (St. Louis). All reagents were of the highest purity available and were not purified further.

Purification of Proteins. Acetone-induced liver microsomes were prepared after treatment of 2-kg male New Zealand rabbits with 1% (vol/vol) acetone in drinking water for 7 days. The microsomal cytochrome P450 content was 2.9 nmol/mg protein. Rabbit NADPH-cytochrome P450 reductase was purified by a published method (4). Full-length CYP2E1 was purified from liver microsomes of acetone-treated rabbits and Δ3-29 CYP2E1 was expressed and purified as described (5-7). All enzymes were electrophoretically homogeneous on 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8).

Analytical Measurements. Absorbance spectra were recorded on a Shimadzu UV3000 Spectrophotometer (Shimadzu Co., Japan). Cytochrome P450 levels were quantitated using an extinction coefficient of $E_{452\text{-}490}$ = 80 mM $^{-1}$cm^{-1} for the reduced CO-complexed minus reduced form (6). NADPH-P450 reductase was quantitated using the extinction coefficient, E_{456nm} = 21.4 mM^{-1}cm^{-1} (9). NADPH-cytochrome c reductase activity was measured at 20°C as described (2). Electron transfer to P450 was monitored at 20°C with a Dionex stopped-flow spectrophotometer exactly as described earlier (2). The DLPC-reconstituted system used in the experiments with pure P450 forms was prepared as described earlier (10). Briefly, P450 and reductase were mixed with DLPC vesicles (DLPC/protein=80/1, P450/reductase=10/1) and incubated for 1 hour at 20°C before initiation of the reaction. The presence of 4-methylpyrazole is required for the stabilization of Δ3-29 CYP2E1 during the purification, and the reconstituted preparations of full-length and Δ3-29 CYP2E1 contained this compound at a level of 0.86 μM. In a separate set of experiments it was shown that this concentration of 4-methylpyrazole did not affect the reduction kinetics of CYP2E1.

RESULTS AND DISCUSSION

Recently we reported that charge interactions disrupt formation of the NADPH-cytochrome P450 reductase/cytochrome P450 electron transfer complex, and suggested that the complex is formed by the attraction of complementary hydrophobic patches on the hemoprotein and the flavoprotein (1-3). Although electron transfer from NADPH-cytochrome P450 reductase

Table 1. Influence of ionic strength on the reduction of cytochrome P450 in rabbit acetone-induced microsomes [a]

Composition of the medium	k_{fast} (s^{-1})	k_{slow} (s^{-1})	Percent as fast phase
10 mM sodium phosphate	0.04	0.01	27
100 mM sodium phosphate	0.11	0.02	40

[a]Acetone-induced rabbit liver microsomes were suspended to 1μM cytochrome P450 in 10 mM or 100 mM sodium phosphate buffer, pH 7.4. Cytochrome P450 reduction was monitored as described in Methods.

to rat CYP2E1 is inhibited by low ionic strength (1), the influence of ionic strength on rabbit liver CYP2E1 reduction has not been tested. Treatment of rabbits with acetone induces CYP2E1 in liver microsomes (11). As shown in Table 1, reduction of cytochrome P450 in acetone-induced rabbit liver microsomes is stimulated by increasing ionic strength. This stimulation is in agreement with the facilitation of electron transfer to two other rabbit cytochrome P450 forms, CYP1A2 and CYP2B4, upon neutralization of electrostatic interactions (1-3). Analysis of the computer alignment of cytochrome P450 sequences (12) revealed a number of conserved hydrophobic regions within the hemoprotein molecule that may be involved in such interaction. All microsomal cytochrome P450 forms have a highly hydrophobic amino-terminal region due to retention of the signal peptide. This amino-terminal sequence of cytochrome P450 has been suggested to participate in the interaction with NADPH-cytochrome P450 reductase (13). We examined the possibility of the involvement of the amino-terminal region in the reductase-cytochrome P450 interaction using truncated Δ3-29 rabbit CYP2E1. Deletion of amino acids 3-29 removes the first conserved hydrophobic segment, termed S1 by Nelson and Strobel (12). It also removes the major part of the hydrophilic connecting region between S1 and the second conserved hydrophobic region, S2 (residues 32-48). After deletion of amino acids 3-29 CYP2E1 remains predominantly membrane-bound (5, 7). Furthermore, it is also active in the metabolism of ethanol, *N*-nitrosodiethylamine, aniline and *p*-nitrophenol (7), indicating that protein folding is largely unaffected by deletion of amino acids 3-29. Deletion of amino acids 3-29 could affect NADPH-cytochrome P450 reductase-cytochrome P450 interaction in three possible ways:

1. ***If the initial hydrophobic segment of CYP2E1 is an essential hydrophobic patch involved in the binding of NADPH-cytochrome P450 reductase to the cytochrome P450,*** its removal would be expected to impair reductase-cytochrome P450 interactions. At low ionic strength, when charge repulsion between the hemoprotein and the flavoprotein is most

pronounced, a decreased number of the hydrophobic binding sites should result in much slower reduction of the truncated cytochrome P450 compared to the full-length enzyme.

2. If ***Lys-24*** (located in the hydrophilic region between the amino-terminal conserved hydrophobic domain, S1, and the second hydrophobic region, S2) ***is involved in charge repulsion*** between the reductase and the hemoprotein, its deletion would decrease the repulsion and facilitate protein-protein interaction. In this case reduction of Δ3-29 CYP2E1 at low ionic strength would be expected to proceed faster than the reduction of the full-length enzyme.

3. If ***the NH_2-terminal hydrophobic and the subsequent hydrophilic region are not essential*** for NADPH-cytochrome P450 reductase-cytochrome P450 interaction, the rates of Δ3-29 CYP2E1 and full-length CYP2E1 reduction would be identical at low and high ionic strength.

To evaluate the changes induced by deletion of amino acids 3-29 we monitored reduction of full-length and truncated CYP2E1 at low (10 mM sodium phosphate, pH 7.4) and high ionic strength (100 mM sodium phosphate, pH 7.4). Results of a typical experiment are presented in Fig. 1. Both full-length and Δ3-29 CYP2E1 were fully reducible by the NADPH-cytochrome

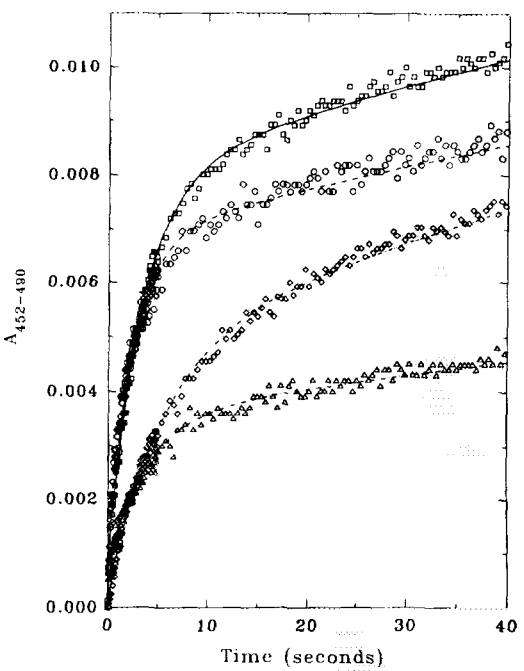

Fig. 1. Influence of ionic strength on the reduction of full-length rabbit CYP2E1 and Δ3-29 CYP2E1. Full-length CYP2E1 or Δ3-29 CYP2E1 was reconstituted with the reductase in DLPC (cytochrome P450/reductase = 10/1, DLPC/protein = 80/1 mole/mole) and suspended to 0.5 μM cytochrome P450: full-length CYP2E1 in 10 mM (-·-△) and 100 mM (--○) sodium phosphate, pH 7.4; Δ3-29 CYP2E1 in 10 mM (-···◇) and 100 mM (——□) sodium phosphate, pH 7.4. Cytochrome P450 reduction was monitored as described in Methods. Each curve is a mean of six individual records. Lines are drawn according to the kinetic parameters presented in Table 2.

Table 2. Influence of ionic strength on the reduction of full-length rabbit CYP2E1 and Δ3-29 CYP2E1 [a]

Cytochrome P450 used	Composition of the medium	k_{fast} (s^{-1})	k_{slow} (s^{-1})	Percent as fast phase
Full-length CYP2E1	10 mM sodium phosphate	0.20	0.002	16
	100 mM sodium phosphate	0.39	0.003	45
Δ3-29 CYP2E1	10 mM sodium phosphate	0.19	0.005	29
	100 mM sodium phosphate	0.37	0.005	40

[a]Full-length CYP2E1 or Δ3-29 CYP2E1 was reconstituted with the reductase in DLPC (cytochrome P450/reductase = 10/1, DLPC/protein = 80/1 mole/mole) and suspended in the indicated medium to 0.5 μM cytochrome P450. Cytochrome P450 reduction was monitored as described in Methods.

P450 reductase under both conditions. In agreement with our earlier observations (2), reduction of cytochrome P450 proceeded faster at higher ionic strength. Interestingly, reduction of the Δ3-29 truncated form was less inhibited by the lower ionic strength than the full-length enzyme (Fig. 1). Increasing the ionic strength from 10 mM sodium phosphate to 100 mM sodium phosphate stimulated reduction of the full-length CYP2E1 (Table 2). The fast phase rate constant increased two-fold (from 0.20 s^{-1} to 0.39 s^{-1}). The fraction of the cytochrome P450 reduced in the fast phase of the reaction increased from 16% to 45%, while the rate constant of the slow phase of the reduction was much less affected (Table 2). The influence of the ionic strength on the reduction of Δ3-29 CYP2E1 was similar to that observed for the full-length enzyme. An increase in the concentration of the sodium phosphate buffer from 10 mM to 100 mM increased the rate constant of the fast phase of Δ3-29 CYP2E1 reduction two-fold (from 0.19 s^{-1} to 0.37 s^{-1}). The fraction of the hemoprotein reduced in the fast phase of the reaction increased from 29% to 40%, while the rate constant of the slow phase of the reduction was unchanged (Table 2). The rate constants of the fast phase of Δ3-29 CYP2E1 agree well with the values obtained for the full-length CYP2E1. Interestingly, at low ionic strength a higher percent of the truncated cytochrome was reduced in the fast phase compared to the full length enzyme. The rate constant of the slow phase of Δ3-29 CYP2E1 reduction at either ionic strength was also higher than that observed for the full-length CYP2E1.

As mentioned above, if the initial hydrophobic portion of the CYP2E1 is one of the essential hydrophobic patches responsible for the formation of the reductase-cytochrome P450 complex, its deletion would be expected to impair CYP2E1 reduction. The neutralization of

electrostatic interactions at increased ionic strength did not reveal any impairment of the Δ3-29 CYP2E1 reduction in comparison to that of the full-length enzyme, indicating that the amino-terminal hydrophobic region is not essential for the interaction of CYP2E1 with the reductase. On the contrary, reduction of the Δ3-29 CYP2E1 was less inhibited by the low ionic strength. It will be of interest to determine whether Lys-24 is involved in the inhibitory electrostatic interaction with NADPH-cytochrome P450 reductase.

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