Role of Acidic Residues in the Interaction of NADPH-Cytochrome P450 Oxidoreductase with Cytochrome P450 and Cytochrome c*

Anna L. Shen and Charles B. Kasper
From the McArdle Laboratory for Cancer Research, Medical School, University of Wisconsin, Madison, Wisconsin 53706

(Received for publication, August 7, 1995, and in revised form, September 14, 1995)

Site-directed mutagenesis of the acidic clusters 207Asp·Asp·Asp209 and 213Glu·Glu·Asp215 of NADPH-cytochrome P450 oxidoreductase demonstrates that both cytochrome c and cytochrome P450 interact with this region; however, the sites and mechanisms of interaction of the two substrates are clearly distinct. Substitutions in the first acidic cluster did not affect cytochrome c or ferricyanide reductase activity, but substitution of asparagine for aspartate at position 208 reduced cytochrome P450-dependent benzphetamine N-demethylase activity by 63% with no effect on $K_{m}^{P450}$ or $K_{m}^{NADPH}$. Substitutions in the second acidic cluster affected cytochrome c reduction but not benzphetamine N-demethylase or ferricyanide reductase activity. The E213Q enzyme exhibited a 59% reduction in cytochrome c reductase activity and a 47% reduction in $K_{m}^{c}$ under standard conditions ($\times 0.27$ m potassium phosphate, pH 7.7), as well as a decreased $K_{cat}^{c}$ at every ionic strength and a shift of the salt dependence of cytochrome c reductase activity toward lower ionic strengths. The E214Q substitution did not affect cytochrome c reductase activity under standard conditions, but shifted the salt dependence of cytochrome c reductase activity toward higher ionic strengths. Measurements of the effect of ionic strength on steady-state kinetic properties indicated that increasing ionic strength destabilized the reductase-cytochrome $c^{3-}$ ground state and reductase-cytochrome c transition state complexes for the wild-type, E213Q, and E214Q enzymes, suggesting the presence of electrostatic interactions involving Glu213 and Glu214 as well as additional residues outside this region. The ionic strength dependence of $K_{cat}/K_{m}^{c}$ for the wild-type and E213Q enzymes is consistent with the presence of charge-pairing interactions in the transition state and removal of a weak ionic interaction in the reductase-cytochrome c transition-state complex by the E214Q substitution. The ionic strength dependence of the E213Q enzyme, however, is not consistent with a simple electrostatic model. Effects of ionic strength on kinetic properties of E213Q suggest that substitution of glutamine stabilizes the reductase-cytochrome $c^{3-}$ ground-state complex, leading to a net increase in activation energy and decrease in $K_{cat}$. Glu213 is also involved in a repulsive interaction with cytochrome c $c^{3-}$. Cytochrome c $K_{d}$ for the wild-type enzyme was $82.4 \mu M$ at $118 \mu M$ ionic strength and $10.8 \mu M$ at $749 \mu M$ ionic strength; similar values were observed for the E214Q enzyme. Cytochrome c $K_{d}$ for the E213Q enzyme was $17.6 \mu M$ at $118 \mu M$ and $15.7 \mu M$ at $749 \mu M$ ionic strength, consistent with removal of an electrostatic repulsion between the reductase and cytochrome c $c^{3-}$.

The microsomal and nuclear envelope flavoprotein NADPH-cytochrome P450 oxidoreductase (P450R) (NADPH:ferredoxin protein oxidoreductase, EC 1.6.2.4) catalyzes electron transfer from NADPH to the cytochromes P450 (1) and other microsomal proteins (2–4), as well as to nonphysiologic electron acceptors such as cytochrome c, ferricyanide, menadione, and dichlorophenolindophenol (5, 6). There is a substantial body of information on the structure and mechanism of this multienzyme enzyme (7, 8), as well as on its gene structure and regulation (8–10). Although crystals of P450R have been obtained (11), the crystal structure has not been solved. FMN, FAD, and NADPH-binding domains of P450R have been identified by sequence comparisons with flavoproteins of known three-dimensional structure (8, 12, 13) and site-directed mutagenesis has identified amino acids necessary for binding of FMN and NADPH (14–16). The orientation of the nicotinamide and FAD isoalloxazine rings has been shown to be exo, with transfer of the pro-R hydrogen of NADPH to FAD (17). The kinetic mechanism with the substrate cytochrome c is nonclassical (two-site) Ping Pong, with the reaction of cytochrome c at the electron acceptor site being Ping Pong at high ionic strength and random sequential at low ionic strength (18, 19).

Less is known about the substrate binding sites of P450R. A number of electron-transfer complexes are stabilized by electrostatic interactions which play a role both in complex formation and in orienting the two redox centers (20–22); however, evidence also exists for involvement of multiple hydrophobic and van der Waals interactions (23–25). Chemical modification and cross-linking studies (26–29) have provided evidence for electrostatic interactions between P450R and its substrates. For example, neutralization of carboxyl groups on P450R by 1-ethyl-3-(3-dimethylaminopropyl)carboimidide (EDC) has been shown to inhibit both cytochrome c reductase activity and cytochrome P450-dependent monooxygenase activity, with no effect on electron transfer to ferricyanide or cytochrome b$_{5}$ (27–29). Modification of Lys$_{382}$ of CYP2B4 with fluorescein isothiocyanate has been shown to inhibit reductase-dependent but not cumene-hydroperoxide-dependent monooxygenase activity (30) and Shimizu et al. (31) have identified by site-directed mutagenesis seven lysyl and/or arginyl residues in CYP1A2 which affect cytochrome P450-dependent monooxygenase activity. In contrast, studies on the ionic strength dependence of P450 reduction argue against charge-pairing between P450R and cytochrome P450 (32–34).

In attempts to identify specific side chain interactions between P450R and substrate, Nisimoto (26) characterized an EDC cross-linked complex between P450R and cytochrome c, where a lysyl residue in cytochrome c was covalently linked to an acidic residue in the region between residues 200–220 of the reductase. This region contains two clusters of acidic amino acids.
acids, Asp207, Asp208, and Glu213, and conserved residues at positions 204, 216, 219, 220, and 221. Comparison of the rat reductase sequence with that of Desulfovibrio vulgaris flavodoxin reveals identity at positions 208, 215, and 219 as well as the conservation of an acidic residue at position 214. The results presented here focus on the effects of introduction of the corresponding amides for the acidic residues in these two clusters, changes which produce minimal steric effects but remove charge-pairing interactions and introduce the potential for hydrogen-bonding.

Table I shows that substitution of asparagine for Asp208 affects primarily the interaction of P450R with cytochrome P450, as evidenced by a 63% reduction in the P450-dependent benzphetamine N-demethylase activity and no effect on cytochrome c and ferricyanide reduction. Similar changes at either positions 207 or 209 had no effect on the catalytic activity with cytochrome P450, cytochrome c, or ferricyanide. The activity of the double mutant D207N/D208N was similar to that of D208N. The drop in N-demethylase activity was accompanied by a decrease in $k_{cat}$ of 55% for D208N and 66% for D207N/D208N, with no change in $K_m$. No significant changes in $k_{cat}$ or $K_m$ were seen in any of the cluster I single mutants.

In contrast to the results seen with cluster I substitutions, mutations in the second acidic cluster affected interaction of the reductase with cytochrome c but not with cytochrome P450 (Table III). None of the cluster II substitutions affected P450-dependent N-demethylase activity; however, under standard assay conditions (0.27 M potassium phosphate, pH 7.7), cytochrome c reductase activity of the E213Q mutant was decreased by 59%, E214Q, D215N, D207N/E214Q, and D207N/D215N exhibited normal catalytic activities, while the cytochrome c reductase activities of the D207N/E213Q and E213Q/E214Q/D215N mutants were decreased by 70%. As with cluster I mutations, ferricyanide activity was not affected by any of the cluster II mutations.

Each of the cluster I and cluster II single mutants and mutants carrying two or three substitutions had the expected flavin stoichiometry, i.e. 1 mol of FMN and 1 mol of FAD per mol of protein. Replacement of all six acidic residues with their corresponding amides, however, produced a protein with the normal complement of FAD but only 0.4 mol of FMN/mol of protein. The specific activity of this six-place mutant was 10% of wild type and was significantly less than that of E213Q and E213Q/E214Q/D215N. This reduced activity is probably the result of structural perturbations affecting FMN binding.

Examination of the kinetic properties of the cluster II mutants in 0.27 M potassium phosphate, pH 7.7, shows that the decreased specific activity of the E213Q mutant was accompanied by a 60% decrease in $k_{cat}$ and a 47% decrease in $K_m$ (Table IV). Similar decreases were observed in each of the
not affected by any of the cluster II mutations. Substitution at the E214Q and D207N/E214Q mutants, with no change in wild-type and mutant enzymes are shown in Fig. 2. When ionic strength was varied by increasing the concentration of KCl (Fig. 2), wild-type P450 and benzphetamine NADPH-Cytochrome P450 Oxidoreductase Substrate Binding Site (for NADPH), and 50 μM NADPH (for K_mcat), with varying amounts of NADPH or cytochrome c. Reactions were preincubated at 28 °C for 2 min and initiated by addition of NADPH. Values are mean ± S.D. (n).

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| Protein   | Cytochrome c reductase activitya | P450-dependent benzphetamine N-demethylase activityb |
|-----------|----------------------------------|-----------------------------------------------------|
|           | K^{NADPH}_M (μM) | k_{cat} (min^-1) | K^{NADPH}_M (μM) | k_{cat} (min^-1) |
| Wild-type | 6.4 ± 1.0 (3) | 21.1 ± 2.5 (3) | 4704 ± 152 (3) | 0.24 ± 0.01 (4) | 88 ± 9.1 (4) |
| D207N     | 8.3 ± 0.6 (4) | 26.0 ± 2.3 (3) | 5160 ± 400 (4) | 0.23 ± 0.02 (4) | 92 ± 13 (3) |
| D208N     | 8.4 ± 0.6 (3) | 24.2 ± 5.8 (3) | 5256 ± 208 (3) | 0.25 ± 0.05 (3) | 40 ± 13 (3) |
| D209N     | 5.8 ± 1.3 (3) | 23.6 ± 4.3 (3) | 5200 ± 136 (3) | 0.17 (2) | 86 (2) |
| D207N/D208N | 6.4 ± 2.2 (3) | 31.2 ± 2.2 (3) | 4992 ± 416 (3) | 0.25 ± 0.03 (3) | 30 ± 4.3 (3) |

a Reactions contained 0.27 mM potassium phosphate, pH 7.7, 65 μM cytochrome c (for K_mcat), and 50 μM NADPH (for K_mcat), with varying amounts of NADPH or cytochrome c. Reactions were preincubated at 28 °C for 2 min and initiated by addition of NADPH. Values are mean ± S.D. (n).

b Values are mean ± S.D. (n).

c | Protein | Specific activity | Cytochrome c | Ferricyanide | P450 |
|--------|------------------|--------------|--------------|-------|
|        | μmol/ min/ mg    | μmol/ min/ mg | μmol/ min/ mg |
| Wild-type | 51.7 ± 6.0 (3) | 87.9 ± 2.2 (3) | 0.95 ± 0.06 (3) |
| E213Q  | 21.3 ± 3.4 (4) | 96.8 ± 4.8 (3) | 0.97 ± 0.07 (3) |
| E214Q  | 55.8 ± 5.4 (3) | 99.6 ± 18 (4) | 0.92 ± 0.07 (3) |
| D215N  | 44.2 (2) | 118 (1) | 1.0 (1) |
| D207N/E213Q | 15.4 ± 0.5 (4) | 84.1 ± 11 (3) | 1.03 ± 0.02 (3) |
| D207N/E214Q | 65.1 ± 7.0 (3) | 105 ± 13 (3) | 0.87 ± 0.2 (3) |
| D207N/D215N | 44.2 (2) | 105 (2) | 0.81 (1) |
| E213Q/E214Q/D215N | 15.4 ± 1.1 (3) | 98.0 ± 9.9 (3) | 0.96 ± 0.03 (3) |
| D207N/D208N/D209N/E213Q/E214Q/D215N | 5.0 ± 0.1 (3) | ND | ND |

a Reactions contained 0.27 M potassium phosphate, 65 μM cytochrome c, and 50 μM NADPH.

b Reactions contained 0.27 M potassium phosphate, 500 μM potassium ferricyanide, and 100 μM NADPH.

c Activity is expressed as nanomoles of formaldehyde produced per min per μg reductase.

d ND, not determined.

| Protein | Cytochrome c reductase activitya | P450-dependent benzphetamine N-demethylase activityb |
|--------|----------------------------------|-----------------------------------------------------|
|        | K^{NADPH}_M (μM) | k_{cat} (min^-1) | K^{NADPH}_M (μM) | k_{cat} (min^-1) |
| Wild-type | 6.4 ± 1.0 (3) | 21.1 ± 2.5 (3) | 4704 ± 152 (3) | 0.24 ± 0.01 (4) | 97 ± 13 (4) |
| E213Q  | 5.2 ± 0.4 (3) | 11.2 ± 0.7 (3) | 1880 ± 176 (3) | 0.28 ± 0.07 (3) | 115 ± 15 (3) |
| E214Q  | 7.1 ± 0.3 (3) | 33.6 ± 4.0 (3) | 4704 ± 712 (3) | 0.23 ± 0.05 (3) | 91 ± 16 (3) |
| D215N  | 6.6 (2) | 19.8 (2) | 4584 (2) | ND | ND |
| D207N/E213Q | 4.3 ± 0.6 (3) | 10.9 ± 0.8 (4) | 1360 ± 88 (4) | 0.19 ± 0.03 (3) | 112 ± 15 (3) |
| D207N/E214Q | 8.4 (2) | 36.6 ± 3.0 (3) | 5642 (2) | 0.28 ± 0.03 (3) | 77 ± 2 (3) |
| E213Q/E214Q/D215N | 3.7 ± 0.4 (3) | 7.5 ± 1.3 (3) | 1440 ± 88 (3) | 0.17 ± 0.05 (3) | 102 ± 12 (3) |
| Six-place | 2.8 ± 0.6 (3) | 9.4 ± 1.0 (3) | 463 ± 35 (3) | ND | ND |

a Reactions contained 0.27 M potassium phosphate, pH 7.7, 65 μM cytochrome c (for K_mcat), and 50 μM NADPH (for K_mcat), with varying amounts of NADPH or cytochrome c. Reactions were preincubated at 28 °C for 2 min and initiated by addition of NADPH. Values are mean ± S.D. (n).

b Values are mean ± S.D. (n).

c D207N/D208N/D209N/E213Q/E214Q/D215N.

d ND, not determined.

mutants carrying the E213Q substitution (D207N/E213Q, E213Q/E214Q/D215N, and D207N/D208N/D209N/E213Q/E214Q/D215N). In contrast, K^Cytc for substitution at Glu213 produced a slight, but statistically insignificant, decrease in k_{cat}. However, K^NADPH for the three (E213Q/E214Q/D215N) and six-place (D207N/D208N/D209N/E213Q/E214Q/D215N) mutants was significantly decreased to 58 and 44%, respectively, of wild type.

The effects of ionic strength on the catalytic activities of the wild-type and mutant enzymes are shown in Fig. 2. When ionic strength was increased by increasing the concentration of potassium phosphate (Fig. 2A), maximum cytochrome c reductase activity was observed at an ionic strength of approximately 452 mM for the wild-type enzyme, 399 mM for the E213Q enzyme, and 678 mM for the E214Q enzyme. Maximum activity of the E213Q/E214Q/D215N mutant was observed at 502 mM (not shown). When ionic strength was varied by increasing the concentration of KCl (Fig. 2B), wild-type k_{cat} increased to a maximum in the range between 208 and 479 mM, while E213Q k_{cat} was maximal at 208 mM and declined thereafter. k_{cat} for the E214Q enzyme was maximal at ionic strengths greater than 479 mM. While k_{cat} of the E213Q enzyme was only 28% of wild type at 749 mM, it was the same as that of wild type at low ionic strength.
The current study examines the role of two clusters of acidic amino acids, located in the amino-terminal half of P450R, in substrate recognition. The results indicate that the sites and mechanisms of interaction are distinct for the two substrates, as well as for the oxidized and reduced forms of cytochrome c.

Asp$^{208}$ of cluster I affects P-450 catalyzed N-demethylation while Glu$^{213}$ of cluster II influences cytochrome c reduction.
The ionic strength dependence of wild-type and E214Q reductase-cytochrome c interactions is consistent with removal of a weak electrostatic interaction in the E214Q mutant (Fig. 3). In contrast, the ionic strength dependence of E213Q does not fit these models without the introduction of additional terms, either as a result of an unfavorable conformation of the electron transfer complex or as a consequence of altered interactions with reduced cytochrome c.

Comparison of the effects of ionic strength on the kinetic properties of the wild-type, E213Q, and E214Q enzymes permits the identification of the reaction intermediates affected by changes in salt concentration and by removal of either the P450 or cytochrome c. High ionic strength increased the free energy of binding of wild-type (Fig. 4). For the wild-type enzyme, increasing ionic strength increased the free energy of binding for both the ground state and transition state complexes, consistent with the presence of electrostatic interactions between P450 and cytochrome c. High ionic strength increased the binding energy of the ground state complex more than that of the transition state, leading to a net decrease in activation energy and the observed increase in $k_{cat}$. This is an example of the use of the free energy of binding of the enzyme-substrate complex.
complex to increase the rate of catalysis (46). It is likely that this mechanism also underlies the inhibition of cytochrome c reduction by polyols observed by Vosnesensky and Schenkman (32, 33). Similarly, removal of a weak interaction between Glu$^{213}$ and cytochrome c has a larger destabilizing effect on the P450-cytochrome c ground state complex than on the transition state complex, leading to a net increase in $k_{cat}$ at high ionic strength.

Replacement of Glu$^{213}$ with glutamine had no effect at low ionic strength; however, at higher ionic strengths, the ground state complex was more stable than that of the wild-type enzyme, leading to a net increase in activation energy and decrease in $k_{cat}$. Glu$^{213}$ in the wild-type enzyme may interact weakly or not at all with cytochrome c; however, glutamine at position 213 must be able to form hydrogen bonds with cytochrome c which act to stabilize the P450-cytochrome c$^{2+}$ complex in a conformation which is less favorable for electron transfer.

Although Glu$^{213}$ does not interact strongly with oxidized cytochrome c, cytochrome c$^{2+}$ $K_{D}$ of the wild-type enzyme decreased with increasing ionic strength, consistent with the presence of an electrostatic repulsion between P450R and cytochrome P450 (46). It is likely that this mechanism also underlies the inhibition of cytochrome c reduction by polyols observed by Vosnesensky and Schenkman (32, 33). Similarly, removal of a weak interaction between Glu$^{213}$ and cytochrome c has a larger destabilizing effect on the P450-cytochrome c ground state complex than on the transition state complex, leading to a net increase in $k_{cat}$ at high ionic strength.

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The free energy changes associated with the D208N, E213Q, and E214Q substitutions range from nil to 1.3 kcal/mol, substantially less than those commonly associated with loss of an ion binding. This may be because these substitutions do not affect rate-limiting steps in benzphetamine demethylation or because an electrostatic interaction has been replaced with a hydrogen bonding interaction (47), as has been proposed for binding of human adrenal ferredoxin to P450 (51). Free energy changes of this magnitude are also to be expected if multiple electrostatic, hydrophobic, and van der Waals interactions contribute to binding. Mutagenesis of catonic residues of P450 sperm (23) and cytochrome $b_{5}$ (24) which are involved in charge-pairing interactions with putidaredoxin and cytochrome $b_{5}$ reductase, respectively, produced binding energy changes similar to those observed here, and it was proposed that a single electrostatic interaction made a relatively small contribution to overall binding. The altered ionic strength dependence of the E213Q mutant may be an indication of an increased contribution from these other interactions.

In summary, these results demonstrate that residues located in two acidic clusters identified by chemical cross-linking experiments have specific effects on the interaction of P450 with cytochrome c and cytochrome P450. Cytochrome c and cytochrome P450 interact with separate residues in this region. Arg$^{208}$ of P450 influences electron transfer to cytochrome P450 with no effect on binding. Although additional factors are necessary for electron transfer to cytochrome P450 (31, 32–34, 52), the ionic strength data indicate that electrostatic interactions do predominate in the formation of the P450-cytochrome c$^{2+}$ complex (Fig. 3) (6, 52). Glu$^{214}$ interacts weakly with cytochrome c and Glu$^{213}$ is involved in a repulsive interaction with reduced cytochrome c. Glu$^{213}$ may not form contacts with oxidized cytochrome c; however, replacement of Glu$^{213}$ with glutamine may introduce new hydrogen bonding interactions which stabilize the reductase-cytochrome c complex in an altered conformation where electron transfer is less efficient. The effects of ionic strength on the kinetic properties of these mutants point toward multiple charge-pairing, hydrophobic, and repulsive interactions involving these residues and other, as yet unidentified residues, which may also play a role in electron transfer from P450R to its substrates.
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J. Biol. Chem. 1995, 270:27475-27480.
doi: 10.1074/jbc.270.46.27475

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