Negatively Charged Anabaena Flavodoxin Residues (Asp\textsuperscript{144} and Glu\textsuperscript{145}) Are Important for Reconstitution of Cytochrome P450 17α-Hydroxylase Activity*  

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Catalysis by microsomal cytochromes P450 requires the membrane-bound enzyme NADPH-cytochrome P450 reductase (P450 reductase), which transfers electrons to the P450 heme via a flavodoxin-like domain. Previously, we reported that *Escherichia coli* flavodoxin (Fld), a soluble electron transfer protein, directly interacts with the analogous domain in P450 reductase, we have examined whether flavodoxins can serve as useful models of the analogous domain in P450 reductase, we have examined the FNR-Fld system from the cyanobacterium *Anabaena*. Mutagenesis of two acidic *Anabaena* Fld residues (D144A and E145A) significantly decreased flavodoxin-supported P450c17 progesterone 17α-hydroxylase activity. Specifically, D144A exhibited only 15% of the activity of wild-type Fld, whereas the adjacent mutation, E145A, caused a 40% loss in activity. P450-dependent hydrogen peroxide/superoxide production by wild-type FNR-Fld was measurably higher than that generated by FNR-D144A or FNR-E145A, indicating that the mutations do not lead to P450 heme-mediated electron uncoupling. Interestingly, the D144A and E145A mutants bind with equal or even greater affinity to P450c17 than wild-type Fld. Furthermore, these mutations (D144A and E145A) actually increased cytochrome c reductase activity (35 and 100% higher than wild type). *Anabaena* Fld residues Asp\textsuperscript{144} and Glu\textsuperscript{145} align closely with rat P450 reductase residue Asp\textsuperscript{208}, which has been shown by mutagenesis to be important in electron transfer to P4502B1 but not to cytochrome c (Shen, A. L., and Kasper, C. B. (1995) *J. Biol. Chem.* 270, 27475–27480). Thus, these residues in flavodoxins and P450 reductase appear to have similar functions in P450 recognition and/or electron transfer, supporting the hypothesis that flavodoxins represent valid models for the FMN-binding domain of P450 reductase.

Flavodoxins (Flds)\textsuperscript{1} found in microorganisms and certain algae, comprise a group of low molecular weight, acidic, water-soluble flavoproteins containing 1 mol of noncovalently bound FMN/mol of protein (1). The FMN cofactor functions as the redox center of flavodoxins, allowing these proteins to serve as low potential electron shuttles in several important, biologically diverse reactions. Examples of Fld-supported enzymes include Acetobacter (2) and Klebsiella (3) nitrogenases, which generate ammonia from nitrogen; *Anabaena* (4) ferredoxin-NADP\textsuperscript{+} reductase (FNR), which reduces NADP\textsuperscript{+} to NADPH; and *Escherichia coli* (5) methionine synthase, which methylates homocysteine to form methionine.

Primary amino acid sequence comparisons of various flavodoxins to known proteins have revealed similarities that may be structurally and functionally important. Examples include amino acid sequence homology between flavodoxins and the FMN-binding domains of nitric oxide synthase (6), cytochrome P450BM-3 (7, 8), and NADPH-cytochrome P450 reductase (P450 reductase) (9, 10). Most conserved residues in alignments between Flds and P450 reductase, for instance, lie within regions that bind FMN. In addition, clusters of negatively charged residues exist in these proteins, which have been proposed to be involved in the recognition of specific positively charged residues on their corresponding redox partners (11–14).

Mutagenesis of residues within two negatively charged clusters of rat P450 reductase (Asp\textsuperscript{207}-Asp-Asp\textsuperscript{209} (cluster I) and Glu\textsuperscript{211}-Glu-Asp\textsuperscript{215} (cluster II) by Shen and Kasper (15) has demonstrated that these clusters are distinct from one another with respect to their interaction with the electron acceptors cytochrome P450B21 and cytochrome c (cyt c). In particular, substitution of Asn for Asp\textsuperscript{208} significantly decreased the rate of P4502B1-catalyzed benzphetamine N-demethylation without affecting cyt c reduction, whereas substitution of Gln for Glu\textsuperscript{211} decreased the rate of cyt c reduction without altering P4502B1 N-demethylation activity. In the course of investigating the ability of a FNR-Fld system from *Anabaena* to support P450c17-catalyzed 17α-hydroxylation of progesterone, we have found a strong similarity between the results obtained with two mutant Flds, D144A and E145A, and the results from the study of cluster I in P450 reductase. These mutants, in combination with FNR, are considerably less efficient than wild-type Fld in reconstituting P450c17 activity, whereas conversely, their ability to reduce cyt c has been increased. Importantly, *Anabaena*

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\textsuperscript{1} The abbreviations used are: Fld, wild-type flavodoxin; FNR, ferredoxin (flavodoxin)-NADP\textsuperscript{+} reductase; cyt c, cytochrome c; P450 reductase, NADPH-cytochrome P450 reductase; P450c17, 17α-hydroxylase/17,20-lyase cytochrome P450.
Fld residues Asp\textsuperscript{144} and Glu\textsuperscript{145} correspond to Asp\textsuperscript{208} and Asp\textsuperscript{209} respectively, in rat P450 reductase. Spectrophotometric binding experiments indicate that mutagenesis of Asp\textsuperscript{144} or Glu\textsuperscript{145} to alanine has no effect or actually decreases the apparent \( K_a \) of these Flds for P450c17 relative to wild-type Fld. This result is consistent with the Shen and Kaspers observation that the \( K_a \) of P450 reductase for P4502B1 was not changed in Asn\textsuperscript{208} even though electron transfer was significantly affected either directly or indirectly. From these results, we propose that the study of flavodoxin-P450 interactions and electron transfer may yield important information about analogous P450 reductase/P450 processes.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis of Anabaena Fld**—Site-directed mutagenesis of Anabaena Fld was performed as described previously (16).

**Expression and Purification of Recombinant Protein**—Wild-type Anabaena flavodoxin was purified as described previously (17), as was Anabaena FNR (18). Bovine P450c17 was expressed and purified from E. coli (19).

**Assay for P450c17 Progesterone Hydroxylase Activity**—Purified bovine P450c17 (1.3 \( \mu \)M) and Anabaena FNR (0.2 \( \mu \)M) were preincubated (25 °C for 2 min) with varied amounts of Anabaena Fld in reconstitution buffer (10 mM potassium phosphate, pH 7.4, 25 mM NaCl, 10% glycerol, 0.1 mg/ml dilauryl-t-cysteine, 3 mM glucose 6-phosphate, 0.6 units/ml glucose-6-phosphate dehydrogenase) containing \([\text{[H-}}\text{Fe}^2\text{]}\)- and unlabeled progesterone (100,000 cpm and 50 \( \mu \)M, respectively). Reactions (200 \( \mu \)l) were initiated with NADPH (0.3 mM final concentration) and terminated after 10 min by vortexing with 2 ml of chloroform. Following evaporation of the organic phase, steroids were separated by TLC, visualized by UV light, and quantitated by scintillation counting.

**Assay for Cytochrome c Reductase Activity**—50 \( \mu \)l of horse heart cytochrome \( c \) (7.5 mg ml\textsuperscript{-1}), 10 \( \mu \)l of NADPH (6 mM) and 5 \( \mu \)l of FNR (2.6 \( \mu \)M) were mixed with different quantities of Tris-HCl 50 mM, pH 8, in a spectrometer cuvette (maintained at 25 °C). The reaction was initiated by adding concentrated mutant or wild-type Fld (final volume = 500 \( \mu \)l). The absorbance change was followed at 550 nm using 20 mM \( \text{Fe}^3\text{-}\text{c} \) as the extinction coefficient of reduced cyt \( c \).

**Spectral Analysis of Fld Binding to P450c17**—Spectral changes due to titration of P450c17 with wild-type and mutant Flds (initial concentrations = 360–443 \( \mu \)M) using tandem cuvettes were recorded between 350–450 nm as described previously (19) except [P450c17] = 5.0 \( \mu \)M, [dilauryl-t-cysteine-phosphatidylcholine] = 50 \( \mu \)M, and [progesterone] = 50 \( \mu \)M. Fe\textsuperscript{2+}-CO minus Fe\textsuperscript{3+} difference spectra of both the sample and reference cuvettes were measured after each titration to confirm P450c17 stability during the assay.

**Ferroammonium Sulfate/KSCN Oxidation Assay**—P450c17 reactions were performed as described above except \([\text{H}]\)progesterone and, in some cases, P450c17 were omitted. Background \( \text{H}_2\text{O}_2\)/superoxide levels for each sample were measured after the addition of water instead of NADPH. Trichloracetic acid (3% w/v, 100 \( \mu \)l) was used to stop each reaction. Samples were put on ice for 20 min and centrifuged at 10,000 \( \times \) g for 10 min, and the supernatant was removed. A solution of 7.2 mM ferroammonium sulfate in 0.7M KSCN (90 \( \mu \)l) was added to each reaction as well as to hydrogen peroxide standards. After 15 min at room temperature, the absorbance of each sample was determined at 480 nm (20).

**Materials**—[\(\text{H}\)]progesterone was obtained from Amersham Corp. Progesterone was purchased from Steraloids, Inc. TLC plates were purchased from EM Separations. All other reagents and supplies were purchased from Sigma or VWR Scientific.

**RESULTS AND DISCUSSION**

After our initial observations that E. coli FNR and Fld can serve as a reductase system for bovine P450c17 (19), we were interested in determining whether similar proteins from microorganisms other than E. coli would support P450c17 catalytic activity. As shown in Fig. 1, P450c17 progesterone 17a-hydroxylase activity was also found to be dependent on Anabaena Fld in the presence of Anabaena FNR, P450c17, and NADPH. The maximum rate of P450c17 turnover with Anabaena FNR-Fld under these conditions is 2–3-fold higher than with the E. coli system but approximately 10-fold lower than with a 1:1 molar ratio of recombinant rat P450 reductase.

![Fig. 1. Reconstitution of P450c17-dependent progesterone (Prog) 17\(\alpha\)-hydroxylase activity with Anabaena FNR in the presence of wild-type and mutant Anabaena flavodoxins. Points represent the average of three separate experiments. WT, wild type.](image-url)
sites are essential for redox partner recognition (14). Resolution of the crystal structure of Anabaena flavodoxin to 1.4 Å (27) has localized these regions within the protein. Specifically, the first group (Asp123, Asp126, Asp129) lies between two short consecutive 8-sheets (85 and 86b) within a long (20 amino acid) loop present in all long chain flavodoxins, whereas the second group (Asp144, Glu145) is present at the C-terminal end of 86b as part of a turn region (refer to Figs. 3 and 4).

Four mutants, one from the first group (D126A), two from the second group (D144A and E145A), and one from neither group (E67A), have been tested for their ability to transfer electrons to P450c17 from FNR (Fig. 1). The most profound effect occurs with the D144A mutant, which exhibits only 15% wild-type activity over a range of 1–15 μM flavodoxin. Similar mutagenesis of Glu145 (E145A) also results in significantly lower (by 40%) P450c17 activity. Residues Asp144 and Glu145 closely align with rat P450 reductase residue Asp208 (Fig. 4) which, when replaced by asparagine, diminished the ability of this enzyme to support P4502B1 by 63% (28). Substitution of Asp126 of Anabaena Flavodoxin with an alanine had little effect on the ability of this protein to donate electrons to P450c17. The glutamic acid residue at position 67 present in helix a2 (Fig. 3) is positioned more distantly from the FMN isoalloxazine ring than the other three residues. Changing this residue to an alanine causes a 25% loss in activity in comparison to wild-type Flavodoxin.

The roles of these mutations in the Fld-dependent NADPH-cyt c reductase activity of Anabaena FNR are significantly different from their effects on P450 activity (Fig. 2). The D144A and E145A Flavodoxin mutants exhibited greater activity than wild-type (35% and 100% respectively), whereas D126A is 25% less efficient that the wild-type protein. These results are consistent with previous experiments showing increased rates of electron transfer from illuminated spinach photosystem I to the D144A and E145A Flavodoxin mutants (16). Laser flash photolysis techniques were used in this case to measure the direct rate of electron transfer between the photosynthetic reaction center and the Flavodoxin. Deletion of a negative charge at position 67 produced a drastic change in the ability of this protein to transfer electrons to cyt c, as indicated by the absence of saturation kinetics at increasing E67A Flavodoxin concentrations.

This polarity in activities (P450c17 hydroxylase activity versus cyt c reductase activity) observed with D144A and E145A is not unprecedented in studies of electron transfer proteins. For example, site-directed mutagenesis of two Anabaena ferredoxin surface amino acid residues (Phe65 and Glu94) dramatically decreased (by 4 orders of magnitude) electron transfer to Anabaena FNR (28) but actually enhanced the reverse reaction by 1.5–2-fold, as measured by NADPH-cyt c reductase activity (29).

Alignment of the 85 loop region of Anabaena Flavodoxin with the corresponding regions within E. coli Fld and rat cytochrome P450 reductase revealed a conserved negatively charged site within the 85-sheet structure of Anabaena Flavodoxin (Fig. 4). This alignment takes into account the 20–21 residue loop insertion found in all long chain flavodoxins. From this, it would be predicted that the atomic structure of the trypsinized rat P450 reductase revealed a conserved negatively charged site within the 85b-sheet structure of Anabaena Flavodoxin (28) but actually enhanced the reverse reaction by 1.5–2-fold, as measured by NADPH-cyt c reductase activity (29).

In contrast, Asp144 and Glu145 of Anabaena Flavodoxin appeared to play key roles in P450 reductase activity as evidenced by 85% and 40% losses in activity upon mutation to alanine. Questions remained, however, regarding the ability of these mutants to bind and electrochemically couple to P450c17. To address the issue of binding, spectrophotometric changes in P450c17 upon flavodoxin binding were recorded and apparent Ks values calculated (Fig. 5). The absorbance changes measured upon titration with wild-type and mutant Flavodoxins were indicative of a low-to-high spin transition of the P450c17 heme iron as observed with E. coli flavodoxin (19). Elimination of the negatively charged residues at positions 67, 144, or 145 of Anabaena Flavodoxin did not disrupt the ability of these mutants to associate with P450c17. In fact, the apparent Ks value for D144A is 3-fold less than that of wild-type Flavodoxin. The results of these equilibrium binding experiments between the oxidized Flavodoxins and P450c17 may be reflective of the dissociation rate (k_d) of these proteins.
during catalysis. Thus, after transfer of an electron from semiquinone Fld to P450c17, if $k_{\text{cat}}$ is limiting, the steady-state rate of progesterone 17-hydroxylation would be expected to decrease as in the case of D144A (and perhaps E145A). Indeed, loss of these charged residues may reduce charge repulsion, a process found to be important for P450 reductase-P4501A2 and P450 reductase-P4502B4 interactions (34, 35). Alternatively, elimination of either the Asp$^{144}$ or Glu$^{146}$ carboxyl group may result in a flavodoxin-P450c17 complex whose conformation is more rigid and consequently less favorable for electron transfer relative to wild-type Fld. The need for flexibility of electron transfer complexes has been proposed for other redox proteins including cyt c/cyt c peroxidase (30), cyt c/cyt c oxidase (31), cyt c/cytochrome $b_5$ (32), and Anabaena ferredoxin/FNR (33). As evidenced by the almost 4-fold increase in apparent $K_r$, replacement of Asp$^{146}$ with alanine may indirectly enhance charge repulsion, although not significantly enough to affect electron transfer.

Since D144A and E145A Flds did not appear deficient in their ability to bind P450c17 nor to reduce cyt c, we decided to test whether these mutants were more uncoupled to P450c17 than wild-type Fld. One measure of uncoupling of the reaction would be given by the leakage of electrons toward molecular oxygen by either FNR, Fld, and/or P450c17 during catalysis. An assay in which oxidation of Fe(II) to Fe(III) is followed would indicate the amount of reactive oxygen species, mostly hydrogen peroxide and superoxide, being generated in the mixture. We assume that, under the conditions of the assay, superoxide would protonate upon termination of the reaction with trichloroacetic acid (see "Experimental Procedures") to produce HO$_2^-$ which would oxidize Fe(II). From Fig. 6, it is clear that in the absence of P450c17, FNR-D144A generates twice the amount of reactive oxygen as FNR-Fld. Presumably, this is a result of increased uncoupling in the transfer of electrons from FNR to D144A or simply electron leakage from the D144A semiquinone to generate superoxide. This could in part explain the observed 35% increase in cyt c reductase activity, since superoxide is able to directly reduce cyt c. It should be noted, however, that when HO$_2^-$ reacts with Fe(II), HO$_2^-$ is formed and protonated to generate H$_2$O$_2$, which then oxidizes another Fe(II). Thus, the values in Fig. 6 cannot be precisely quantified in terms of the number of electrons lost to oxygen. Non-enzymatic reduction of cyt c by superoxide (maximum rate $= 60$ min$^{-1}$, Fig. 6), however, could not account for the total observed cyt c reductase activity (100 min$^{-1}$, Fig. 6), suggesting that electrons are not being transferred to the heme iron. This is consistent with the loss in P450c17 activity (Fig. 1). In comparison, P450-dependent H$_2$O$_2$/superoxide production by FNR-E67A and FNR-D126A were essentially identical to that seen with FNR-Fld. These data are also consistent with the small changes in P450c17 catalytic activities (Fig. 1) in that electron transfer to the P450 heme iron leads not only to substrate turnover but also to uncoupling to reduce molecular oxygen. Finally, the 40%
loss in P450c17 activity observed with FNR-E145A is accompanied by a comparable decrease in the amount of P450-generated reactive oxygen relative to wild type.

In conclusion, the results presented here support the hypothesis that different structural features are required for flavodoxin to interact with a variety of different reaction partners (as has also been suggested for ferredoxin (16)). For example, mutagenesis of specific amino acid residues in ferredoxin produces a drastic loss in the ability of this protein to exchange electrons with FNR but not with illuminated photosynthetic membranes (16). The fact that different amino acid residues are required for the interaction with one reaction partner but not with another does not necessarily indicate that the electron transfer protein uses different regions to accomplish this, as has been proposed for plastocyanin (36). Presumably, different sites on the surface of flavodoxin make contacts with different partners, as suggested by the contrasting results reported here with cyt c and P450c17. The consistency between the effects of mutagenesis of corresponding regions within Anabaena flavodoxin and rat P450 reductase (15) strongly suggests that these analogous domains are very similar, and as a result, further study of flavodoxins may lead to a greater understanding of how these proteins interact with their multiple redox partners, including cytochromes P450.

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