Site-specific Mutations in Human Ferredoxin That Affect Binding to Ferredoxin Reductase and Cytochrome P450<sub>sec</sub>*

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Ferredoxins found in animal mitochondria function in electron transfer from NADPH-dependent ferredoxin reductase (Fd-reductase) to cytochrome P450 enzymes. To identify residues involved in binding of human ferredoxin to its electron transfer partners, neutral amino acids were introduced in a highly conserved acidic region (positions 68–86) by site-directed mutagenesis of the cDNA. Mutant ferredoxins were produced in Escherichia coli, and separate assays were used to determine the effect of substitutions on the capacity of each mutant to bind to Fd-reductase and cytochrome P450<sub>sec</sub> and to participate in the cholesterol side chain cleavage reaction. Replacements at several positions (mutants D68A, E74Q, and D86A) did not significantly affect activity, suggesting that acidic residues at these positions are not required for binding or electron transfer interactions. In contrast, substitutions at positions 76 and 79 (D76N and D79A) caused dramatic decreases in activity and in the affinity of ferredoxin for both Fd-reductase and P450<sub>sec</sub>; this suggests that the binding sites on ferredoxin for its redox partners overlap. Other substitutions (mutants D72A, D72N, E73A, E73Q, and D79N), however, caused differential effects on binding to Fd-reductase and P450<sub>sec</sub>, suggesting that the interaction sites are not identical.

We propose a model in which Fd-reductase and P450<sub>sec</sub> share a requirement for ferredoxin residues Asp-76 and Asp-79 but have other determinants that differ and play an important role in binding. This model is consistent with the hypothesis that ferredoxin functions as a mobile shuttle in stereodivergent electron transfer, and it is considered unlikely that a functional ternary complex is formed.

The animal mitochondrial ferredoxins are small (~14 kDa), acidic proteins that contain a single [2Fe–2S] cluster. They function as central components in electron transfer from NADPH-dependent ferredoxin oxidoreductase (Fd-reductase) to cytochrome P450 enzymes. These mitochondrial cytochrome P450 enzymes are involved at key steps in a variety of processes, including the first step in steroid hormone biosynthesis, the conversion of cholesterol to pregnenolone catalyzed by the cholesterol side chain cleavage cytochrome P450 (P450<sub>27</sub>). Two different mechanisms have been proposed for ferredoxin-mediated electron transfer from Fd-reductase to P450<sub>sec</sub>. In one model, ferredoxin acts as an electron shuttle, initially forming a complex with Fd-reductase, dissociating after accepting an electron, and then associating with and transferring the reducing equivalent to P450<sub>sec</sub>. This role for ferredoxin as a mobile electron carrier in the cholesterol side chain cleavage reaction is supported by kinetic evidence that maximal activity can be achieved at significantly less than 1 reductase/P450<sub>sec</sub> when ferredoxin is saturating (1), by potentiometric data that indicate that dissociation of ferredoxin from Fd-reductase is reduction-induced (2), and by spectroscopic results that suggest sequential formation of 1:1 complexes between ferredoxin and Fd-reductase and between ferredoxin and P450<sub>sec</sub> (1, 3). In an alternative model, the three proteins function as a ternary complex.

Support for this model comes from reports that a complex with 1:1:1 molar ratio of the three redox partner proteins was isolated (4) and that chemically cross-linked binary complexes between Fd-reductase and ferredoxin can transfer electrons to P450<sub>sec</sub> (5, 6). One distinction between the two models is that ternary complex formation requires distinct binding sites on ferredoxin for Fd-reductase and P450<sub>sec</sub>. The associations between Fd-reductase, ferredoxin, and P450<sub>sec</sub> are strongly dependent on ionic strength (2), indicating an important role for electrostatic interactions. The amino acid sequences of mitochondrial ferredoxins from human (7), cow (8), pig (9), and chick (10) indicate that the protein is highly acidic (pI 4.0–4.5) and contains two conserved regions of 6 or more acidic residues that are uninterrupted by basic amino acids (positions 27–47 and 68–86, Fig. 1). Chemical modification studies using bovine ferredoxin have indicated that acidic residues within the second region (positions 68–86) are important for binding to Fd-reductase (11). However, due to lack of specificity in chemical labeling, it was not possible to unequivocally identify the specific residues involved or to distinguish which residues were important for association with Fd-reductase and which contribute to P450<sub>sec</sub> binding.

In an effort to define the Fd-reductase and P450<sub>sec</sub>-binding domains of human ferredoxin, we have utilized site-directed mutagenesis to introduce mutations into the cloned cDNA. These mutations resulted in ferredoxins with neutral amino acid substitutions at acidic positions in the region containing residues 68 through 86. Mutant proteins were prepared using an expression system developed for producing human ferredoxin in Escherichia coli (12) and were analyzed in vitro for their ability to support P450-catalyzed cholesterol side chain cleavage, to associate with Fd-reductase, and to bind P450<sub>sec</sub>.
FIG. 1. Acidic regions of mitochondrial ferredoxins. The number-
shing shown corresponds to amino acid positions in the mature proteins follow-
ing removal of the mitochondrial import signal sequences. Charged residues within Acidic Regions 1 and 2 are indicated. Amino acid sequences are deduced from cDNA sequences reported for the human (7), cow (8), pig (J. L. Omdahl; personal communication), and chick (10) ferredoxins and are supported by partial amino acid sequence data in the case of the human (17), cow (38, 39), and pig (9) proteins. Cysteine residues proposed (40) to be coordinated to the [2Fe-2S] cluster (positions 46, 52, 55, and 92) are shown in bold type and are enclosed in boxes.

EXPERIMENTAL PROCEDURES
Restriction endonucleases were obtained from Boehringer Mann-heim. Tag DNA polymerase was from U. S. Biochemical Corp. Re-
agents for bacterial growth media were from GIBCO, and other chemicals were from Sigma.

Mutagenesis—Synthetic 20-mer oligonucleotides containing single
or double point mutations were obtained from either Operon Tech-
nologies (Alameda, CA) or the UCR Biotechnology Instrumentation Facility (University of California, Riverside, CA). Synthetic oligonucleo-
tides were phosphorylated on 14% denaturing polyacrylamide gels (13) followed by electroelution (14). Oligonucleotide-directed mutagenesis was performed on ferredoxin DNA vector pHFD1 (12) using either a gapped-duplex method (Boehringer Mannheim mutagenesis kit) or a polymerase chain reaction method developed by Nelson and Long (15). In both cases, mutated DNA was treated with BamHI and HindIII and subcloned into the expression vector pMb3 (16). The resulting plasmids were sequenced using a double-stranded dyeoxy method (13) to confirm that mutagenesis was limited to the predicted sites.

Preparation of Mutant Ferredoxins—Expression in E. coli (12) and
purification of ferredoxin protein (17) was as previously described,
except that transformed MZ-1 cells were induced at an As*, of 0.35,
which had been previously shown to be optimal for mutant expression (17) and recombinant (12) human ferredoxins are function-
ally equivalent to bovine adrenal ferredoxin (adrenodoxin) when
reconstituted in vitro with bovine adrenal Fd-reductase (adrenodoxin
reductase) and bovine adrenal P450.,

Cytochrome c reduction was assayed in 33 mM potassium phos-
phate, pH 7.2 at room temperature. Reaction mixtures contained 10
nm Fd-reductase, 20 µM horse heart cytochrome c (Sigma type VI)
and a NADPH-regenerating system. Reduction was monitored spec-
rophotometrically at 550 nm, and activity was determined assuming
ΔA550 = 20 (mM-cm)-1. Under these conditions, Fd-reductase is lim-
iting; rates observed for wild type and mutant ferredoxins were
independent of the amount of cytochrome c present when tested at
cytchrome c concentrations ranging from 10 to 40 µM.

Spectral titrations were performed as described (24) using 0.6 µM
P450., in 33 mM potassium phosphate, pH 7.2, 0.1% Tween 20, and
70 µM cholesterol at room temperature. Specified amounts of ferre-
doxin were added from concentrated stock solutions such that final
dilutions of reaction mixtures were less than 4%, and dilutions were cor-
rected for in calculating difference spectra. The concentrations of free ferredoxin were calculated using the following equation

ferredoxin, = ferredoxin, - (ΔA/ΔAsat) × P450.

Values of kinetic parameters were determined by least squares linear
regression analysis of the data from two separate experiments.

RESULTS

Production of Mutants—Two types of amino acid substitutions
were made for acidic residues between positions 68 and 86 as shown below.

| 70 | 75 | 80 | 85 | 90 |
|----|----|----|----|----|
| L-D-A-1-7-D-E-F-N-D-M-L-D-L-A-Y-G-L-T-D-R-S-F-L- | A | N | Q | N | A |
| A | A | A | A |

In most cases, aspartate or glutamate was replaced with the corresponding
amino acids to maintain polarity at that position and to attempt to
minimize structural perturbations. Alanine, although smaller and nonpolar,
was used to replace acidic amino acids at some positions as an alter-
native neutral residue. With the exception of substitutions at Glu-74, each of
the expressed mutants yielded soluble ferredoxin that incor-
porated an iron-sulfur cluster in vivo. Replacement of glutamate
at position 74 with either alanine (E74A) or glutamine (E74Q), however,
resulted in formation of insoluble apoprotein in E. coli. By reconstitution in vitro with iron and sulfur

Vertebrate Ferredoxin Sequences

| Acidic Region 1 | Acidic Region 2 |
|-----------------|-----------------|
| Human | SSSRDRITVPHQKNGGELTLLRGKGGGLDVLVIDGFDQSGTTLVSFL |
| Bovine | -K- | -Q- |
| Porcine | -DK- | -A- |
| Chick | -M- | -E- |
| -F- | -F- | -M- | -E- |
| -F- | -F- | -M- | -E- | -K- |
| -F- | -F- | -M- | -E- | -K- |

2 for wild type ferredoxin); additional experiments were performed
using ferredoxin mutants D76N and D79N at a concentration of 2 µM. Reaction mixtures contained 25 nm P450.; 30 nm Fd-reductase,
and a NADPH-regenerating system. Incubations were for 5 min at
37 °C in 33 mM potassium phosphate, pH 7.2, 0.1% Tween 20, and
30 µM cholesterol; pregnenolone produced was measured by radio-
immunoassay (18, 23). We have previously shown that both naturally
occurring (17) and recombinant (12) human ferredoxins are function-
ally equivalent to bovine adrenal ferredoxin (adrenodoxin) when
reconstituted in vitro with bovine adrenal Fd-reductase (adrenodoxin
reductase) and bovine adrenal P450.;

For reconstituted ferredoxin, the value ΔA550 was obtained assuming
ΔA550 = 20 (mM-cm)-1. Under these conditions, Fd-reductase is lim-
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In most cases, aspartate or glutamate was replaced with the corresponding
amino acids to maintain polarity at that position and to attempt to
minimize structural perturbations. Alanine, although smaller and nonpolar,
was used to replace acidic amino acids at some positions as an alter-
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resulted in formation of insoluble apoprotein in E. coli. By reconstitution in vitro with iron and sulfur
Human Ferredoxin Mutants

These results suggest that substitutions at these positions did not alter the environment in the immediate vicinity of the [2Fe-2S] cluster. Mutant E74Q exhibited visible CD spectra that were qualitatively similar to wild type ferredoxin in terms of band shape and position of peaks, but it was not possible to accurately determine \( \Delta \theta \) values due to instability of the sample.

Reconstitution Assay—Each mutant was initially tested for the capacity to participate in the cholesterol side chain cleavage reaction by reconstitution of mutant ferredoxins with bovine Fd-reductase and bovine P450. Activity in this assay requires binding of ferredoxin to Fd-reductase and to P450, and productive electron transfer. These experiments were initially carried out using a limiting concentration of ferredoxin (wild type \( K_m/2 \)), in order to ensure that differences in the affinity of mutant ferredoxins for the redox partners would be apparent. The results are summarized in Table I.

The mutants could be classified into three groups based on their specific activity. Mutants D68A and D86A exhibited activity that was similar to that of wild type ferredoxin (>2 ng of pregnenolone/min) indicating that binding and electron transfer were not significantly affected by these substitutions. Mutants D72A, D72N, E73A, and E73Q showed activity that was reduced to slightly less than half that of wild type (1.1–1.4 ng of pregnenolone/min) suggesting that acidic residues at these positions may be important for association of ferredoxin with one or both redox partner proteins. For mutants D76N, D79A, and D79N, activity was too low (<0.1 ng of pregnenolone/min) to be accurately measured at the ferredoxin concentration used in this experiment. In an attempt to measure the specific activity of mutants D76N, D79A, and D79N, an 8-fold higher concentration (2 \( \mu \)M ferredoxin) was tested in a separate experiment; under these conditions, all three mutants exhibited a capacity to support cholesterol side chain cleavage, but with low specific activity (0.42, 0.60, 0.67 ng of pregnenolone/min, respectively). The finding of activity only at elevated ferredoxin concentrations suggests that the reduced activity observed in mutants D76N, D79A, and D79N most likely arises from decreased binding to Fd-reductase.

Table I

| Ferredoxin   | Cholesterol side chain cleavage activity | Fd-reductase cytochrome c reduction assay | P450 catalytic binding assay |
|--------------|-----------------------------------------|------------------------------------------|-----------------------------|
|              | \( nM \) | \( nmol \) cytochrome c reduced/min | \( K_m \) | \( V_{max} \) | \( K_m \) | \( A_{max} \) |
| Wild type    | 2.80   | 17.2 | 0.80 | 0.8 | 0.032 |
| D68A         | 2.47   | 18.3 | 0.79 | 0.7 | 0.028 |
| D72A         | 1.18   | 27.3 | 0.64 | 2.6 | 0.020 |
| D72N         | 1.12   | 24.7 | 0.72 | 2.5 | 0.025 |
| E73A         | 1.49   | 18.3 | 0.77 | 2.9 | 0.025 |
| E73Q         | 1.20   | 18.6 | 0.74 | 3.1 | 0.027 |
| E74Q\(^a\)   | NA\(^b\) | 20.4 | 0.69 | 0.6 | 0.025 |
| D76N         | <0.1  | ~2000 | 0.00 | 5.0 | 0.025 |
| D79A         | <0.1  | ~1900 | 0.64 | 6.6 | 0.020 |
| D79N         | <0.1  | ~2400 | 0.74 | 1.1 | 0.030 |
| D86A         | 2.13   | 17.7 | 0.79 | 0.9 | 0.027 |

\(^a\) Mutant E74Q was unstable, and kinetic parameters were estimated from limited data (see text).

\(^b\) NA, not available because of instability of this mutant at 37 °C.
and/or P450cc and not from a failure to transfer electrons. Due to its instability at 37 °C, it was not possible to characterize mutant E74Q in this assay.

Binding to Fd-reductase—To determine the extent to which mutations affect binding of ferredoxin to Fd-reductase, we utilized an assay involving cytochrome c as an electron acceptor/indicator. Although this reaction does not occur physiologically, it has been widely used to analyze the kinetics of association of ferredoxin with Fd-reductase (2, 11, 17, 25). Under the assay conditions employed, the experimentally derived \( K_a \) values are equivalent to dissociation constants (\( K_d \)) for the Fd-reductase:ferredoxin complex (2). Kinetic results obtained in this assay are plotted in Fig. 3 and are summarized in Table I. At saturating concentrations, all ferredoxins were active, but mutants could be classified into two groups (low and high affinity), based on their apparent \( K_a \) for Fd-reductase. Low affinities were observed for mutants D76N, D79A, and D79N; apparent \( K_a \) values obtained for these mutants (~1.9–2.4 \( \mu \)M) were more than 100-fold greater than wild type. In contrast, \( K_a \) values obtained for mutants D68A, E73A, E73Q, and D86A (17–19 nM) were very close to that determined for wild type ferredoxin (17 nM). Small but reproducible increases in \( K_a \) values were observed for mutants D72A and D72N (25–28 nM), but these differences (~1.4–1.6-fold increase in \( K_a \)) are much less than the >100-fold increases in \( K_a \) values observed for mutants D76N, D79A, and D79N. Only minor differences in \( V_{\text{max}} \) values were observed between mutant ferredoxins in the low affinity group (D76N, D79A, and D79N; range 0.60–0.74 nmol of cytochrome c reduced/min) and those in the high affinity group (D68A, D72A, D72N, E73A, E73Q, and D86A; 0.64–0.79 nmol of cytochrome c reduced/min); this suggests that the reduced activities of mutants D76N, D79A, and D79N are due to decreased binding affinity and not to defects in electron transfer. Mutant E74Q exhibited activity in this assay, but because a limited amount of this unstable mutant was available, only two concentrations were examined. The estimated \( K_a \) and \( V_{\text{max}} \) values for E74Q were similar to values obtained for mutants in the high affinity group (\( K_a \approx 20 \text{ nM}; V_{\text{max}} \approx 0.6 \text{ nmol of cytochrome c reduced/min} \)).

As a further test of electron transfer function, additional experiments were performed in which we added approximately 100-fold molar excess of mutants D76N, D79A, or D79N (1 \( \mu \)M) to reactions containing a limiting amount of wild type ferredoxin (10 nM). The measured activities were additive (data not shown); wild type activity was not inhibited in the presence of any of these low affinity mutants. This result indicates that when D76N, D79A, or D79N binds to Fd-reductase, electron transfer proceeds at a rate similar to that achieved with wild type ferredoxin.

Taken together, these results indicate that Asp-76 and Asp-79 are critical for high affinity binding of ferredoxin to Fd-reductase, whereas Asp-68, Asp-72, Glu-73, Glu-74, and Asp-86 are not essential. None of these acidic residues appear to be specifically required for electron transfer once the complex is formed.

Binding to P450cc—To test whether amino acid substitutions affect binding of ferredoxin to P450cc, we utilized a spectral binding assay. In the presence of detergent and limiting cholesterol, P450cc exists predominantly as the low spin, substrate-free form with a Soret maximum near 417 nm (22). Binding of ferredoxin to P450cc induces cholesterol binding and results in conversion of the cytochrome to a high spin form having a Soret peak near 392 nm (26). Thus, binding of ferredoxin to P450cc can be followed by monitoring difference spectra resulting from the shift in the Soret spectrum. Difference spectra recorded during titration of P450cc with either wild type ferredoxin or mutant D76N are shown in Fig. 4. In both cases, spectral changes corresponding to a shift in P450cc from low to high spin were associated with ferredoxin binding, but much larger changes were induced by wild type ferredoxin than by mutant D76N. The data from these titrations and from similar experiments with other mutants are plotted in Fig. 5; the calculated spectral dissociation constants (\( K_d \)) and \( DA_{\text{max}} \) values are summarized in Table I.

Wild type ferredoxin exhibited a \( K_d \) value of 0.8 \( \mu \)M under the conditions used in this experiment. A range of different

![Cytochrome c Reductase Assay](image-url)

**Fig. 3.** Cytochrome c reductase activity of human ferredoxins. Data are presented from experiments using wild type ferredoxin (●) and mutants D68A (▲), D72A (●), D72N (○), E73A (■), E73Q (□), D76N (△), D79A (▼), D79N (▲), and D86A (○). Samples were assayed using 10 nM bovine ferredoxin reductase and contained 33 mM potassium phosphate, pH 7.2, and 0.1% Tween-20. Instability limited the data available for mutant E74Q (▼), and only two concentrations were reported.

![P450cc-Ferredoxin Binding Difference Spectra](image-url)

**Fig. 4.** Spectrophotometric titrations of bovine P450cc with human ferredoxins. Difference spectra obtained for wild type ferredoxin and mutant D76N. Samples contained 0.6 \( \mu \)M bovine P450cc, 70 \( \mu \)M cholesterol, and 0.1% Tween-20 in 33 mM potassium phosphate, pH 7.2. The concentrations of ferredoxin were 0.4, 0.8, 1.2, and 2 \( \mu \)M for wild type and 1.5, 3, 5, and 8 \( \mu \)M for D76N.
Human Ferredoxin Mutants

FIG. 5. Analysis of P450red spectral titrations. Data are presented from experiments using wild type ferredoxin (●) and mutants D68A (○), D72A (♦), D72N (◇), E73A (■), E73Q (□), D76N (△), D79A (▲), D79N (▼), and D86A (▲). Instability limited the data available for mutant E74Q (◊), and only two concentrations are reported.

More important for interaction with P450red than for association with Fd-reductase.

Only limited measurements were possible for E74Q due to instability of the protein, but the data obtained at two concentrations indicate that this mutant exhibits an apparent binding affinity for P450red similar to that observed for wild type ferredoxin (Kd ~ 0.6 μM). Because only minor differences in binding affinity were also observed for this mutant in the Fd-reductase assay, Glu-74 (like Asp-68 and Asp-86) does not appear to be required for interaction with either Fd-reductase or P450red.

DISCUSSION

Previous efforts to identify specific groups that are involved in electrostatic interactions between ferredoxin and its reductase partners have utilized chemical modification. In ferredoxin, acidic residues appear to be most important; labeling of lysines in bovine adrenal ferredoxin had no effect on binding (27), but modification at acidic residues inhibited binding to Fd-reductase (11). In these experiments, modifications appeared to occur predominantly at ferredoxin positions 74, 79, and 86, but multiple and nonstoichiometric labeling made it impossible to determine the extent to which individual amino acid residues contribute to Fd-reductase binding. Moreover, it is possible that the bulky labeling reagent may have also prevented interactions at neighboring sites (e.g. position 76). These problems are circumvented in the present study by the use of site-directed mutagenesis. As with chemical labeling, however, it is possible that modifications can alter native protein structure. This limitation is illustrated by mutations at ferredoxin position 74 where substitutions of alanine or glutamine for glutamate resulted in proteins with markedly decreased stability. Although mutant E74Q was unstable, it exhibited activity in the Fd-reductase cytochrome c reduction assay and induced a spectral shift in P450red with binding affinity approximating that observed for wild type ferredoxin. These results suggest that although Glu-74 may play some role in maintaining ferredoxin structure, it is not essential for binding to either Fd-reductase or P450red.

The finding that each of the stable mutant proteins exhibit visible absorption and CD spectra indistinguishable from wild type ferredoxin indicates that the mutations did not significantly alter the environment around the [2Fe-2S] center. CD spectra recorded in the far UV indicate that gross changes in polypeptide backbone structure were also absent in these mutants. The atomic structure of an animal ferredoxin has not been solved, and without crystallographic data on wild type and mutant proteins, we cannot rule out small conformational changes; however, the spectroscopic data suggest that large deviations from wild type activity observed among mutants most likely result from neutralization of acidic residues and not changes in protein structure. Preliminary 1H NMR data support this conclusion: no significant differences in well defined aromatic and hyperfine shifted regions were apparent in spectra recorded at 600 MHz for wild type recombinant human ferredoxin and mutants D76N and D79A.

Models for Fd-reductase and P450red binding that are consistent with the biochemical properties of these mutant ferredoxins are shown in Fig. 6. In these models, Asp-76 is critical for binding both Fd-reductase and P450red. A polar residue at position 79 is also important for binding of both Fd-reductase and P450red. These problems are circumvented in the present study by the use of site-directed mutagenesis. As with chemical labeling, however, it is possible that modifications can alter native protein structure. This limitation is illustrated by mutations at ferredoxin position 74 where substitutions of alanine or glutamine for glutamate resulted in proteins with markedly decreased stability. Although mutant E74Q was unstable, it exhibited activity in the Fd-reductase cytochrome c reduction assay and induced a spectral shift in P450red with binding affinity approximating that observed for wild type ferredoxin. These results suggest that although Glu-74 may play some role in maintaining ferredoxin structure, it is not essential for binding to either Fd-reductase or P450red.

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partner recognition and complex formation. The decreased possibility that such modified complexes have no physiological complex has not been demonstrated.

Binding to Fd-reductase and then to D79A, and D79N in the Fd-reductase interaction correspond binding affinities observed for ferredoxin mutants D76N, D79A, and D79N are similar to those employed in the present study (31, 32), but some of the charged residues could also be involved in forming hydrogen bonds (33). The involvement of an acidic residue in hydrogen bonding may account for the differences in P450c complex binding observed for mutants at position 79; replacement of Asp-79 with a nonpolar amino acid (alanine) inhibited P450c complex binding, whereas substitution with the corresponding amide produced only a small increase in $K_c$.

Although the results demonstrate that Asp-76 and Asp-79 are critical for binding of ferredoxin to both Fd-reductase and P450c, it is likely that additional residues play an important role in these associations. The methods employed in this study may be useful for further mapping of Fd-reductase and P450c-binding domains and serve to refine our preliminary models. The specific residues in Fd-reductase and P450c that interact with ferredoxin have not been unequivocally identified, but chemical modification of lysine amino groups in both proteins has been shown to inhibit ferredoxin binding (34–37). Site-specific mutagenesis may also be useful to help identify the complementary charges in Fd-reductase and P450c complex that interact with the acidic ferredoxin residues identified in this study.

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*Estimates of free energy were made using $\Delta \Delta T = -RT \ln \frac{K_m \cdot c}{K_a \cdot c + K_b \cdot c}$, assuming that the experimentally derived $K_a$ and $K_b$ values are equivalent to the true physical dissociation constants ($K_d$ values) for binding of ferredoxin to its redox partners. This has previously been shown to be the case for $K_a$ values obtained in the Fd-reductase cytochrome c reduction assay when using conditions similar to those employed in the present study (2). The spectral dissociation constants ($K_d$) were determined using oxidized ferredoxin, but the reduction state of the iron-sulfur protein has been shown to have little effect on P450c association (31).
Human Ferredoxin Mutants

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