The Role of Cysteine Residues of Spinach Ferredoxin–NADP$^+$ Reductase As Assessed by Site-Directed Mutagenesis†

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ABSTRACT: To investigate the functional role of the cysteine residues present in the spinach ferredoxin–NADP$^+$ oxidoreductase, we individually replaced each of the five cysteine residues with serine using site-directed mutagenesis. All of the mutant reductases were correctly assembled in *Escherichia coli* except for the C42S mutant protein. C114S and C137S mutant enzymes apparently showed structural and kinetic properties very similar to those of the wild-type reductase. However, C272S and C132S mutations yielded enzymes with a decreased catalytic activity in the ferredoxin-dependent reaction (14 and 31% of the wild type, respectively). Whereas the C132S was fully competent in the diaphorase reaction, the C272S mutant flavoprotein showed a 35-fold reduction in catalytic efficiency with respect to the wild-type enzyme (0.4 versus 14.28 μM^{-1} s^{-1}) due to a substantial decrease of k_{cat}. NADP$^+$ binding by the C272S mutant enzyme was apparently quantitatively the same (K_d = 37 μM) but qualitatively different, as shown by the differential spectrum. Stopped-flow experiments showed that the enzyme–FAD reduction rate was considerably decreased in the C272S mutant reductase, along with a much lower yield of the charge-transfer transient species. It is inferred from these data that the charge transfer (FAD·NADPH) between the reductase and NADPH is required for hydride transfer from the pyridine nucleotide to flavin to occur with a rate compatible with catalysis.

Ferredoxin–NADP$^+$ reductase (FNR,[1] EC 1.18.1.2) is the flavoenzyme responsible for storage, as reducing equivalents in NADPH, of the light energy captured through the photosynthetic process in plants and cyanobacteria (Carrillo & Vallejos, 1987; Zanetti & Aliverti, 1991). FNR acts as a transducer between one-electron carriers, i.e., ferredoxin, and two-electron acceptors (NADP$^+$), exploiting the capacity of its prosthetic group (FAD) to be reduced to the semiquinone level by the first electron and then sequentially reduced to the dihydroquinone by the second electron, thus pairing the electrons for hydride transfer to NADP$^+$ (Batie & Kamin, 1984). The sequences of the enzyme from seven different sources are now available, and the three-dimensional structure of the spinach enzyme has been resolved to 2.6-Å resolution (Karplus et al., 1991).

In addition to being a key enzyme in photosynthesis, FNR has been proposed as a model for a family of structurally related enzymes of outstanding interest such as cytochrome P-450 reductase, cytochrome b_5 reductase, and nitrate and sulfite reductases (Karplus et al., 1991). Recently, a new member was added to this family, namely, the nitric oxide synthase (Bredt et al., 1991). Except for FNR, there is not yet a three-dimensional structure for all of these proteins: as a consequence, studies on FNR could give some insight into their mechanism of action. We recently cloned and expressed in *Escherichia coli* the spinach FNR (Aliverti et al., 1990). Two residues, Lys116 and Lys244, shown by chemical modification (Cidaria et al., 1985; Aliverti et al., 1991a; Chan et al., 1985) to be involved in NADP$^+$ binding, were changed to glutamine by site-directed mutagenesis (Aliverti et al., 1991b). Protein engineering enabled us to confirm a role in NADP$^+$ binding for both of the lysines. Lys244 contributes to stabilization of the Michaelis complex with NADP(H), whereas the Lys116 side chain also plays an important role in the further steps of the catalytic cycle, with both K_m and k_{cat} being greatly affected by the mutation.

It seemed worthwhile to investigate the role of the cysteines in FNR by site-directed mutagenesis. One of them has been suggested to be essential for activity on the basis of inactivation by sulfhydryl reagents such as organomercury compounds (Zanetti & Forti, 1969; Keirns & Wang, 1972) and *N*-ethylmaleimide (Forti & Zanetti, 1967; Davis & San Pietro, 1977; Valle et al., 1982). Actually, we have shown that a role for sulfhydryl groups in FNR catalysis on the basis of *N*-ethylmaleimide inactivation can be rule out by the finding that Lys116 was the target of such a modification (Aliverti et al., 1991a). Furthermore, two vicinal thiols were suggested by experiments with iodosylbenzoate to be at the ferredoxin binding site (Valle et al., 1982). Spinach FNR has five cysteines which exist as free thiols: four in the FAD binding domain (42, 114, 132, 137) and one (272) in the NADP$^+$ binding domain. Their positions in the three-dimensional structure are depicted in the computer graphics model shown in Figure 1. We decided to change each cysteine to serine

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[1] Abbreviations: FNR, ferredoxin–NADP$^+$ oxidoreductase; Fd, ferredoxin; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

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FIGURE 1: α-Carbon structure of spinach ferredoxin–NADP+ reductase. The five cysteine residues of the wild-type enzyme are highlighted, with black dots indicating sulfur atoms. The left half of the molecule constitutes the FAD binding domain (the isoalloxazine ring of FAD, which has been omitted for clarity, fills the cavity indicated by a star), and the right part represents the NADP+ binding domain (Karplus et al., 1991).

because this amino acid is considered to be the closest analog of cysteine both sterically and chemically.

Herein we report on the expression of the mutant enzymes in *E. coli* and on the purification and characterization of only four of the five mutants, because FNR-C42S did not assemble as a holoenzyme. As for the four remaining mutants, only FNR-C272S showed an overall decreased catalytic efficiency, whereas FNR-C132S had partially impaired ferredoxin-dependent cytochrome *c* reductase activity but maintained its full diaphorase activity.

Table I: Nucleotide Sequence of Synthetic Oligonucleotides Used for Mutagenesis

amino acid position	synthetic primer[a]	codon change[b]
42	CGTTGGAAGATCTCTTCTTAAC	TGT→TCT
114	CTGTTTCGTTGTCTGTAAAACGACTC	TGT→TCT
132	CAAGGGAGTCAGCTCCAAC	TGC→AGC
137	TTCTTGTCTGACTTGAAACCC	TGT→TCT
272	TTCTACATGTCTGGTCTCAAGGG	TGT→TCT

[a] Base changes are underlined. [b] All codon changes will substitute serine for cysteine.

EXPERIMENTAL PROCEDURES

Horse heart cytochrome *c*, INT, NADP(H), and *p*-(chloromercuri)phenyl sulfonate were obtained from Sigma. Restriction endonucleases and T4 DNA ligase were purchased either from Gibco BRL or Boehringer Mannheim. All other chemicals were of analytical grade.

Oligonucleotide-Directed Mutagenesis. Site-directed mutagenesis of the FNR gene construct was carried out as described (Aliverti et al., 1991b), according to the "gapped duplex" method (Kramer et al., 1984). Oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer and purified by electrophoresis in 15% polyacrylamide gels. The sequence of the five synthetic oligonucleotides used for mutagenesis is reported in Table I. The presence of the desired mutation and the lack of second-site mutations were confirmed by sequence analysis of the entire mutagenized gene. DNA sequencing was performed according to the chain termination method (Sanger et al., 1977) using single-stranded M13mp9 templates. The fragments carrying the mutated FNR genes were recloned in the expression vector pDS12/RBSII,*Sph*I, following the same strategy previously used for the other FNR mutants (Aliverti et al., 1991b), to yield the plasmids pFNR2-C42S, pFNR2-C114S, pFNR2-C132S, pFNR2-C137S, and pFNR2-C272S.

Purification of the Mutant FNR Forms. Wild-type and mutant enzymes were purified from *E. coli* (host strain RRIΔM15) according to the improved purification procedure developed for the isolation of FNR-K116Q and FNR-K244Q (Aliverti et al., 1991b).

Spectral Analyses. Absorption spectra were recorded with a Hewlett-Packard diode array 8452A spectrophotometer equipped with a Hewlett-Packard 89500A ChemStation. The extinction coefficient at 458 nm of the protein-bound flavin was determined by resolving the FAD from the apoprotein and spectrophotometrically quantitating the released FAD. Enzyme solutions (20–30 μM) in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA were used to obtain the spectrum of the FNR forms. Protein samples were denatured by adding sodium dodecyl sulfate to 0.2% final concentration. The FAD release from FNR was complete in a few minutes at 25 °C. Protein and flavin fluorescence were monitored on a Jasco

FP-777 spectrofluorometer. Circular dichroism spectra were recorded using a Jasco J-500A spectropolarimeter.

Steady-State Kinetic Measurements. Cytochrome *c* reductase activity was assayed essentially as published (Zanetti & Curti, 1980). Diaphorase activity was measured with either INT (Zanetti, 1981) or $K_3Fe(CN)_6$ as the acceptor. In the latter case, the activity was assayed in 0.1 M Tris-HCl (pH 8.2) in the presence of the regenerating system for NADPH already described (Gozzer et al., 1977). Steady-state kinetic data were fit to the enzyme mechanism models using the Hewlett-Packard 89512A enzyme kinetics software.

Stopped-Flow Kinetic Measurements. Rapid-reaction kinetics were studied with a Hi-Tech SF-61 stopped-flow spectrophotometer interfaced with a MacIntosh computer. Data collection and analysis were performed using the KISS software (developed by C. Bull and R. Alexander, 1992). The hydride transfer between NADPH and FNR forms was studied by mixing ca. 40 μM enzyme solutions with solutions of NADPH at various concentrations at 4 °C in the stopped-flow apparatus. Reactants were prepared in 50 mM HEPES buffer (pH 7.0) and made anaerobic by bubbling O_2-free nitrogen (NADPH solutions) or by successive evacuation and flushing with O_2-free N_2 (FNR solutions in a tonometer). The reaction was followed at several wavelengths in the range 340–700 nm. When needed, spectra were acquired at a scan rate of 100 nm s^{-1}.

Determination of the Dissociation Constant of the Enzyme–Ferredoxin Complexes. The K_d values of the complexes of the wild-type and mutant enzymes with oxidized ferredoxin were determined by titrating the reductase protein fluorescence with ferredoxin in the same conditions as described previously (Aliverti et al., 1991b).

Inactivation by Organomercury Reagents: Determination of the Dissociation Constant of the Enzyme–NADP$^+$ Complexes. The reactivity of the wild-type and mutant FNR forms toward *p*-(chloromercuri)phenyl sulfonate was studied under essentially the same conditions as in Zanetti and Forti (1969). Approximately 1 μM enzyme was incubated at 25 °C in 50 mM Tris-HCl (pH 8.2) with 30 μM organomercury. At intervals, aliquots were withdrawn and assayed for INT diaphorase activity. To measure the K_d values of the complexes of the enzyme forms with the pyridine nucleotide substrate, enzyme samples were incubated in the presence of NADP$^+$ concentrations ranging from 25 μM to 1 mM. The analysis of the inactivation rate data was performed according to the procedure of Scrutton and Utter (1965).

Determination of the Difference Absorption Spectrum of the Complexes between FNR Forms and NADP$^+$. FNR samples were diluted in 10 mM Tris-HCl (pH 7.7, at 15 °C) to a final concentration of 35–40 μM. After the spectrum was recorded, NADP$^+$ was added at saturating concentration, and the final spectrum was recorded. Difference spectra were computed by subtracting the initial spectra from the final ones, using the Hewlett-Packard 89510A general scanning software. Corrections were made for dilution due to ligand addition.

RESULTS

Isolation of FNR Mutants. All five mutant FNRs were expressed by *E. coli* cells. Only FNR-C132S and FNR-C272S could be purified with a yield comparable to that of the wild-type enzyme. In the case of the FNR-C42S, the protein was lost in the very early stages of purification. The use of a different expression system (manuscript in preparation), in which the FNR-C42S was produced in a large amount as a fusion protein, allowed us to observe that the FNR moiety of the chimeric protein was in the apoprotein form. The FNR-C114S and FNR-C137S mutants had absorption spectra essentially identical to that of the wild-type enzyme and showed a quenched flavin fluorescence as expected. The catalytic properties of these mutants were very similar to those of the wild-type FNR (Table II). The specific activities of FNR-C132S and FNR-C272S were instead substantially different from those of the wild-type enzyme. Thus, a detailed analysis was carried out on the latter two enzyme mutants.

Table II: Specific Activities of the Various FNRs

enzyme form	INT[a] (units/mg)	cyt *c*[b] (units/mg)
wild type	80	110
Cys114Ser	94	99
Cys132Ser	81	34
Cys137Ser	70	96
Cys272Ser	10	15

[a] Diaphorase activity measured with INT as electron acceptor. [b] Ferredoxin-dependent cytochrome *c* reductase activity. Both assays were performed as stated in the Experimental Procedures.

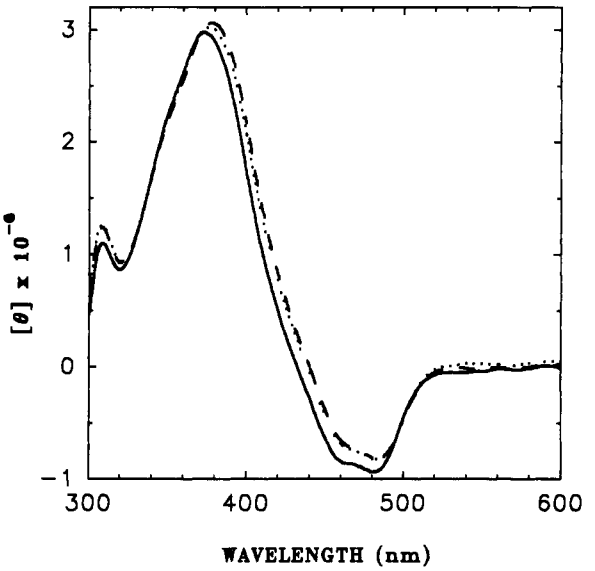

FIGURE 2: CD spectra of C132S, C272S, and wild-type FNRs. The enzymes were diluted to about 50 μM final concentration in 50 mM Tris-HCl (pH 7.4). Spectra were recorded at 20 °C: (—) FNR-C272S; (- - -) FNR-C132S; (···) wild-type FNR.

Spectral Properties. The absorption spectra of both mutant proteins were essentially similar to that of the wild-type enzyme, with only small differences in the case of the C272S form. The main flavin band in the electronic spectrum of the latter flavoprotein was blue-shifted by 2 nm and showed slightly better resolved shoulders. The extinction coefficients at 458 nm of FNR-C132S and FNR-C272S were found to be unchanged with respect to that of the wild-type enzyme (10 300 M^{-1} cm^{-1}) (Zanetti & Aliverti, 1991). In view of the small differences found in the absorption spectrum of FNR-C272S, it was deemed important to monitor more adequately the flavin in the active center by CD and fluorescence measurements. In Figure 2, the CD spectra in the visible range of the two mutants and the wild-type enzyme are compared. Whereas the FNR-C132S spectrum was superimposable upon that of the wild-type enzyme, a blue shift was observed in the FNR-C272S spectrum, which correlates with that found in the absorption spectrum. No significant differences in protein and FAD fluorescence were found for both mutants as compared to the wild-type FNR.

Table III: Kinetic Parameters of the Various FNRs in the Diaphorase Reaction with INT as Acceptor[a]

enzyme form	k_{cat} (e^- (equiv s^{-1}))	K_m^{NADPH} (μM)	K_m^{INT} (μM)
wild type	202 ± 12	49 ± 4	156 ± 12
Cys132Ser	426 ± 21	38 ± 2	238 ± 20
Cys272Ser	30 ± 1	141 ± 6	48 ● 3

[a] Diaphorase activity with INT as substrate was measured as reported in the Experimental Procedures.

Steady-State Kinetics. FNR-C272S showed the same degree of impairment both as diaphorase (with INT as electron acceptor) and as cytochrome *c* reductase (ferredoxin-dependent) (Table II). Although fully active in the diaphorase assay, FNR-C132S retained only about 30% of the cytochrome *c* reductase activity. To gain further insight into the catalytic properties of these FNR forms, steady-state kinetic analyses were performed.

Steady-state kinetic parameters for the INT–diaphorase reaction were obtained by fitting the data to a ping-pong mechanism, and the calculated values are reported in Table III. Serine replacement of Cys132 significantly affected k_{cat}, which was increased ca. 2-fold. The K_m for NADPH was substantially unchanged, so that the catalytic efficiency of the mutant enzyme (k_{cat}/K_m^{NADPH}) was doubled. On the other hand, using $K_3Fe(CN)_6$ as acceptor, the k_{cat} value was found to be similar to that of the wild-type FNR (data not shown). The major effect of the serine replacement of Cys272 was on the k_{cat}. The catalytic rate of the diaphorase reaction was about 15% of that of the wild-type FNR. The values of k_{cat} and K_m^{NADPH} found for FNR-C272S in the ferricyanide reduction were 31 equiv s^{-1} and 77 μM, respectively. The corresponding values for the wild-type flavoprotein were 500 equiv s^{-1} and 35 μM, respectively (Aliverti et al., 1990). If compared to the INT reductase activity, the turnover number of FNR-C272S in the ferricyanide reaction is thus still lower, being ca. 6% of that of the spinach FNR. The differences found with the two acceptors can be explained in terms of different rate-limiting steps (see Discussion).

Rapid Reaction Studies. The time course of flavin reduction in the wild-type and C272S enzymes was studied by the stopped-flow apparatus to verify whether the reduced catalytic rates observed for the mutant FNR could be ascribed to a decreased reduction rate of the FAD prosthetic group by NADPH. The kinetics of reduction of the enzymes by NADPH was determined by following the flavin spectral changes under anaerobic conditions. The concentration of NADPH used (48 μM, 2.6 mol/mol of FAD) ensured pseudo-first-order conditions, since the K_d value of FNR for NADPH has been reported to be ca. 1 μM (Batie & Kamin, 1986). As expected, the wild-type FNR very rapidly formed an intermediate species which has been indicated to be a charge-transfer complex (FNR_{ox}·NADPH) (Massey et al., 1970). The observed decrease of absorption at 460 nm (trace in Figure 3A) was best fit by two exponentials with a fast phase, representing about 24% of the observed decrease with $k_1 >$ 700 s^{-1}, and a second larger phase with k_2 = 106 s^{-1}. Thus, formation of the charge-transfer species (FNR_{ox}·NADPH), which accounted for 56% of the total decrease at A_{460}, was complete in 4–5 ms from mixing ($k_1 >$ 700 s^{-1}), and then reduction of flavin occurred with formation of the charge-transfer species (FNR_{red}·$NADP^+$) as well as other complexes (FNR_{red}·NADPH or FNR_{ox}·$NADP^+$), as reported by Batie and Kamin (1984, 1986).

A value for flavin reduction of 106 s^{-1} is well in keeping with those already published (50 s^{-1} in 0.1 M phosphate (pH 7), 2 °C (Massey et al., 1970) and 206 s^{-1} in 50 mM HEPES (pH 8), 7 °C (Batie & Kamin, 1984)), taking into account the differences in ionic strength, pH, and *T*. In the long-wavelength region (A_{600}, Figure 3), the disappearance of the charge-transfer band seemed monophasic (k = 100 s^{-1}). Furthermore, the slow rate (k_2) decreased at higher NADPH concentration (*i.e.*, 75 s^{-1} at 96 μM NADPH). This has already been reported (Batie & Kamin, 1986). In the reduction of FNR-C272S, the amount of intermediate formed was very low (Figure 3B) and the decrease in FAD absorption was monophasic with k = 7.2 s^{-1}. Again, increasing the NADPH concentration diminished the rate constant of flavin reduction (*i.e.*, 5.7 s^{-1} at 96 μM NADPH).

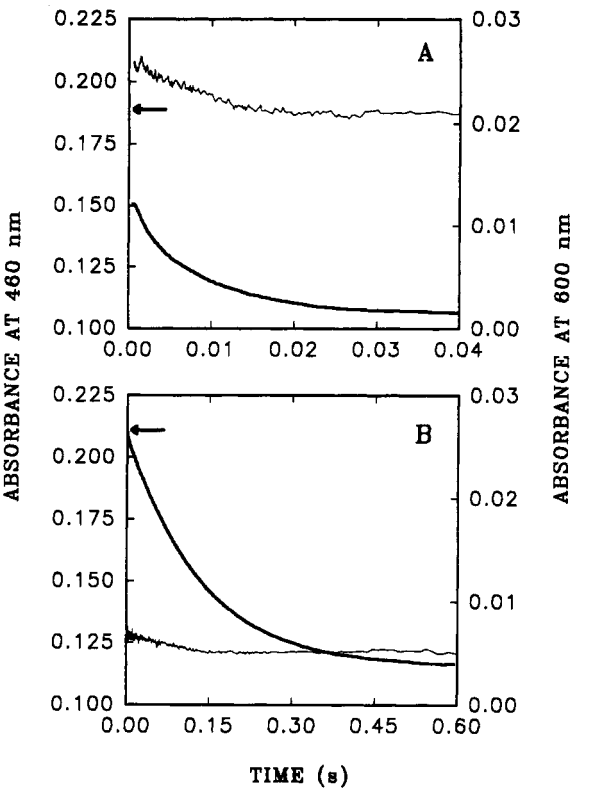

FIGURE 3: Time course of anaerobic reduction of FNR by NADPH: comparison of wild-type enzyme with C272S mutant. Linear plots of A_{460} (darkened line) and A_{600} (thin line) versus time. The arrows indicate the initial absorbance of samples at 460 nm. Reactions were carried out in 50 mM HEPES (pH 7.0) at 4 °C: (A) 38.6 μM oxidized wild-type FNR was mixed with 96 μM NADPH in the stopped-flow apparatus; (B) 40.2 μM FNR-C272S was reacted as above.

Interaction with $NADP^+$. Since Cys272 is in the postulated $NADP^+$ binding site, it was important to study the interaction of the mutant protein FNR-C272S with $NADP^+$. A slight perturbation of the flavin spectrum was recorded only when a concentrated solution of the enzyme was titrated with $NADP^+$ (Figure 4). Apparently, the differential spectrum obtained was similar in shape to that yielded by the wild-type FNR, but it did not change substantially at higher $NADP^+$/FNR ratios, suggesting that saturation was almost attained at the $NADP^+$ concentration used. Thus, to measure the K_d value of the FNR-C272S for $NADP^+$, we had to resort to protection by the ligand toward chemical modification of the enzyme.

p-(Chloromercuri)phenyl sulfonate is a powerful inhibitor of the spinach FNR, and $NADP^+$ affords protection against such inactivation (Zanetti & Forti, 1969). FNR-C272S was the only one of the mutants to show a decreased inactivation rate with the organomercury (ca. 3-fold in comparison to the

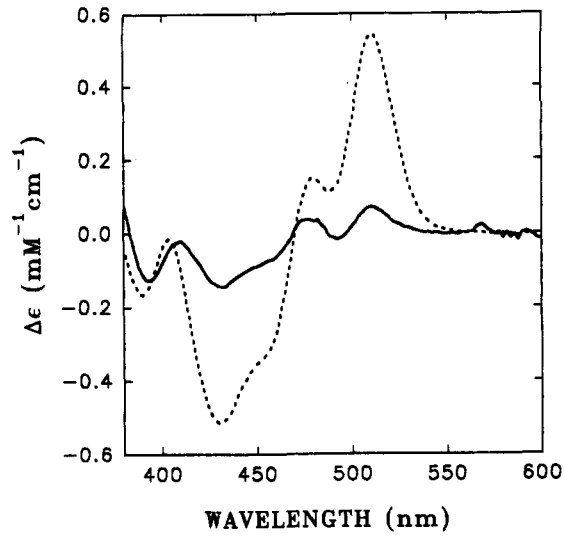

FIGURE 4: Difference absorption spectra induced by $NADP^+$ binding to wild-type and mutant FNRs. Conditions: titrations with $NADP^+$ were performed in 10 mM Tris-HCl (pH 7.7) at 15 °C. Difference spectra of 34.8 μM FNR-C272S (—) and of 39.2 μM wild-type FNR (- - -) were obtained at 1.11 mM $NADP^+$. To facilitate data comparison, the apparent Δε versus wavelength was plotted.

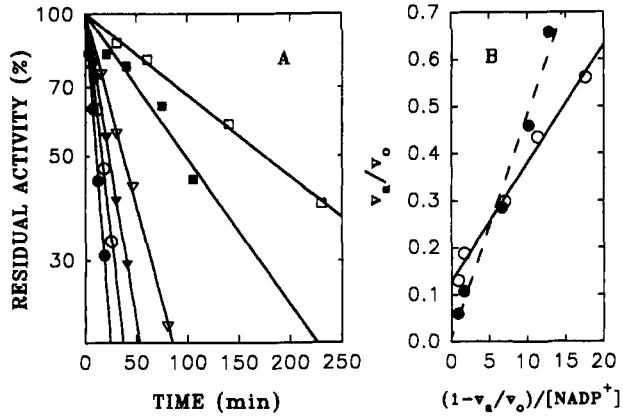

FIGURE 5: Determination of the $NADP^+$ binding constant (K_d) of the FNR-C272S. (A) Time course of inactivation of the enzyme by *p*-(chloromercuri)phenyl sulfonate in the absence and presence of various concentrations of $NADP^+$. Conditions: 1.4 μM FNR-C272S was incubated in 50 mM Tris-HCl (pH 8.2) at 25 °C with 30 μM organomercury. $NADP^+$ concentration in the reaction mixture: (●) none; (○) 27 μM; (▼) 54 μM; (▽) 107 μM; (■) 0.54 mM; (□) 1.07 mM. At the time indicated, an aliquot of the incubation mixture was assayed for INT reductase activity. (B) Calculation of K_d. Data from A (●, - - -) and from an analogous experiment made in the same conditions with wild-type enzyme (○, —) were plotted (Scrutton & Utter, 1965). The slopes of the straight lines yield the K_d values for $NADP^+$ of the two enzymes.

wild-type protein). Also, in the case of this mutant, the presence of $NADP^+$ in the incubation mixture gave substantial protection toward inactivation (Figure 5A). Treatment according to Scrutton and Utter of the data of the experiment in Figure 5A and of an analogous one with the wild-type enzyme allowed us to calculate a K_d for the mutant FNR, which was found to be just slightly larger than that of the wild-type FNR (37 versus 24 μM) (Figure 5B). Thus, mutation of cysteine to serine did not substantially influence the affinity of the enzyme for the substrate $NADP^+$. However, a different mode of binding is suggested by the much lower yield in the typical difference spectrum.

Interaction with Ferredoxin. The interaction of both FNR-C132S and FNR-C272S with oxidized ferredoxin was studied. In the case of FNR-C272S, the aim was to exclude long-range conformational effects in the mutant enzyme which could affect the ferredoxin binding site. On the other hand, since FNR-C132S showed a decreased ferredoxin-dependent activity, it was important to verify whether this effect was due to a lower affinity of the enzyme for the protein substrate. Oxidized ferredoxin forms a complex with FNR which can be monitored through quenching of the protein fluorescence. With both mutant proteins, the extent of quenching reached at ferredoxin-saturating concentrations was the same as that found in the case of the wild-type FNR. Moreover, analysis of the titration curves allowed estimates of the K_d values of the complexes with the different FNR forms; the values found for FNR-C132S and FNR-C272S (0.019 and 0.011 μM, respectively) were very similar to that measured for wild-type FNR (0.015 μM).

DISCUSSION

Our mutagenesis studies point to a specific role in catalysis for just two of the five cysteine residues of FNR: Cys132 and Cys272. The data are in good agreement with the recent reports on the sequences of the enzyme from Anabaena PCC 7119 (Fillat et al., 1990) and that from *Cyanophora paradoxa* [as reported by Schulter and Bryant (1992)]. Accordingly, only Cys132 and Cys272 are conserved in all seven of the FNRs sequenced up to now.

The failure of the FNR-C42S to yield a correctly folded holoenzyme can be rationalized in terms of the three-dimensional structure at high resolution recently published (Karplus et al., 1991). By computer graphics analysis, it can be seen that the environment surrounding the Cys42 side chain is highly hydrophobic. Thus, in this case, the replacement of cysteine with serine was not ideal. The serine side chain, due to its hydrophilic character, would not fit in the hydrophobic pocket, and quite unexpectedly, it destabilizes the entire molecule. It should be noted that, in the *Anabaena* FNR sequence (Fillat et al., 1990), this cysteine is replaced by a hydrophobic residue, a valine. Our data point to a possible misinterpretation of the results of protein engineering when a three-dimensional structure of the protein is not available. Thus, replacement of a residue with different amino acids should be attempted before a structural role for that residue can be claimed. It is anticipated that replacement of the cysteine with an alanine or a glycine will yield a correctly folded holoenzyme. In the case of Cys114 and Cys137 engineering, our choice to change them to serine was an allowed replacement, not only because the mutant FNRs C114S and C137S were correctly assembled and fully active but also because there are natural variants of these mutant forms of the enzyme. The *C. paradoxa* FNR has serine in place of the cysteine 114 (the numbering is that of the spinach enzyme), whereas the *Anabaena* reductase presents a threonine instead of Cys137. Nevertheless, the two mutant FNRs (C114S and C137S) seem less stable than the wild-type protein, suggesting that possibly complementary mutations compensate for the destabilizing effect of the hydroxyl group in the natural variants.

Of the two remaining cysteine mutants, FNR-C132S is the least impaired in catalysis. Actually, the V of the diaphorase reaction was unchanged ($K_3Fe(CN)_6$ as electron acceptor) or even improved (INT reduction). In fact, the k_{cat} of the ferricyanide reaction reflects the rate-limiting step of hydride transfer from NADPH to flavin (Massey et al., 1970). Instead, the cytochrome *c* reduction activity, which requires ferredoxin, was decreased substantially. The partial characterization of the C132S mutant performed up to now allows us only to

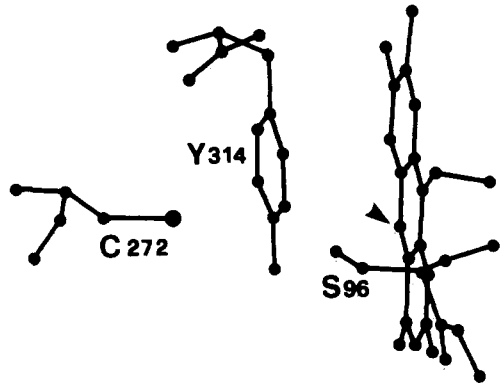

FIGURE 6: Computer graphics drawing of the active center of ferredoxin–NADP$^+$ reductase. A larger dot indicates the sulfur atom of Cys272, and an arrow points to the N_5 position of the isoalloxazine ring of FAD. Other relevant residues (Tyr314 and Ser96) are also shown).

suggest that replacement of Cys132 with serine would affect the kinetic parameters rather than the interaction between the two proteins, because ferredoxin binding to the mutant enzyme as measured by K_d was unchanged. Cys132 is part of the only α-helix (residues 131–137) in the FAD binding domain, and its peptide amide is suggested to be hydrogen-bonded to the pyrophosphate phosphoryl group closer to the isoalloxazine ring (Karplus et al., 1991). The spectral properties of FNR-C132S are identical to those of the wild-type enzyme. Thus, replacement of the SH group with the OH group does not seem to influence the environment of the isoalloxazine ring.

The properties of the FNR-C272S mutant are particularly significant, not only because this cysteine is conserved in all of the FNRs but also because it is a key residue in the NAD(P) binding domain of all of the enzymes belonging to the new family proposed by Karplus et al. (1991). Thus, an understanding of its role in catalysis could also have mechanistic implications for other dehydrogenases. Prior to extensive characterization of the mutant enzyme, several criteria were evaluated to establish that the conformation of the mutant protein had not been substantially altered by the amino acid substitution. These criteria included the protein and FAD fluorescence quenching, indicative of the state of the active-site flavin, and the binding of the substrate (*i.e.*, ferredoxin) remote from the mutational site. All of these parameters were unchanged. Nevertheless, the mutant reductase in its oxidized form showed an electronic spectrum slightly blue-shifted with respect to that of the wild-type enzyme. Thus, the introduction of the OH group in the active site of the reductase clearly seems to perturb the flavin absorption slightly. A possible interference with the stacking interaction between the tyrosine 314 phenol ring and the isoalloxazine (Karplus et al., 1991) (Figure 6) could be ruled out because of the unchanged quenching of FAD fluorescence and even more because of the unchanged intensity and shape of the CD bands in the mutant protein, which support the assumption that the isoalloxazine ring has maintained its proper orientation in the active site, inasmuch as CD spectra will primarily reflect changes in the local environment of the chromophore.

It has been proposed (Karplus et al., 1991) following the glutathione reductase model (Pai et al., 1988) that, by NADP$^+$ binding to the reductase, the side chain of Tyr314 would have to move out of the pocket to leave space for the nicotinamide ring. In the case of the mutant FNR-C272S, binding of NADP$^+$ to the enzyme is taking place with essentially the same K_d but with a yield in the difference spectrum about 10 times lower than that with the wild-type reductase. Thus, either the orientation of the nicotinamide ring in the stacking interaction with the FAD isoalloxazine moiety in the mutant protein is such that perturbation of the flavin spectrum is similar to that obtained with the wild-type FNR, but of lower intensity, or the correct positioning possibly is elicited about 10% of the time.

Furthermore, a similar proposal can be put forward when analyzing the rapid kinetics data obtained by mixing oxidized enzyme and NADPH. The transient spectrum of the charge transfer between NADPH and oxidized flavin observed with the wild-type reductase was difficult to measure quantitatively in the mutant reductase. A low level of it (about 10% of that expected) cannot be excluded, and its presence will fit with the proposal that only through the charge-transfer interaction can subsequent hydride transfer to the flavin take place. Alternatively, the mutant is unable to form the charge transfer, and flavin reduction occurs with an apparent rate constant much lower than that measured with the wild-type enzyme. The extent of reduction of the mutant FNR by NADPH is the same as that obtained with the wild-type reductase, suggesting that the redox potential of the bound flavin was not substantially changed by the mutation.

The k of 7.2 s^{-1} obtained at 4 °C with FNR-C272S in the rapid-reaction studies is fully in agreement with the V measured in the ferricyanide reaction at 25 °C (k_{cat} = 31 s^{-1}), taking into account the difference in pH and temperature. Both values are ca. 6% of the wild-type enzyme values. The mutant enzyme thus has a catalytic efficiency (k_{cat}/K_m^{NADPH}) 35-fold lower than that of the wild-type reductase (0.40 versus 14.28 μM^{-1} s^{-1}). In the case of the INT reductase reaction, the wild-type enzyme is less efficient than with ferricyanide, and the mutant FNR is only 20-fold less efficient with respect to the wild type because the rate-limiting step is the oxidation of the flavin by INT and not its reduction by NADPH.

Clearly, destabilization of the charge-transfer species in the mutant flavoprotein resulted in a much decreased rate of flavin reduction and of overall catalysis, suggesting that indeed the charge-transfer complexes in FNR are precursors of hydride transfer between flavin and pyridine nucleotide. Batie and Kamin cast doubt upon the involvement of the charge-transfer species in the physiological reaction because they could not see these species during turnover (Batie & Kamin, 1984).

Site-directed mutagenesis of the equivalent cysteine (Cys273) in cytochrome b_5 reductase has been recently reported (Shirabe et al., 1991). The C273S mutant enzyme was fully active, whereas a C273A mutation brought about a 5-fold decrease in k_{cat} as well as in catalytic efficiency. Thus, in the case of cytochrome b_5 reductase, the hydroxyl side chain is an allowed substitution for the sulfhydryl group. Even the replacement of cysteine with alanine produced a smaller effect than that measured in the ferredoxin–NADP$^+$ reductase C272S mutant. This would point to a slightly different topography of the active center in cytochrome b_5 reductase with respect to FNR.

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