Is the NAD(P)H:Flavin Oxidoreductase from Escherichia coli a Member of the Ferredoxin-NADP+ Reductase Family?

EVIDENCE FOR THE CATALYTIC ROLE OF SERINE 49 RESIDUE*

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The NAD(P)H:flavin oxidoreductase from Escherichia coli, Fre, is a monomer of 26.1 kDa which catalyzes the reduction of free flavins by NADPH or NADH. The flavin reductase Fre is the prototype of a new class of flavin reductases able to transfer electrons with no prosthetic group. It has been suggested that the flavin reductase could belong to the ferredoxin-NADP+ reductase (FNR) family, on the basis of limited sequence homologies. A sequence, conserved within the ferredoxin-NADP+ reductase family and present in the flavin reductase, is important for recognition of the isoalloxazine ring. Within this sequence, we have mutated serine 49 of the flavin reductase into alanine or threonine. $k_{cat}$ value of the S49A mutant was 35-fold lower than $k_{cat}$ of the wild-type enzyme. Determination of real $K_d$ values for NADPH and lumichrome, a flavin analog, showed that recognition of the flavin is strongly affected by the S49A mutation, whereas affinity for the nicotinamide cofactor is only weakly modified. This suggests that serine 49 is involved in the binding of the isoalloxazine ring. Moreover, the $K_d$ value for 5-deazariboflavin, in which the N-5 position of the isoalloxazine ring has been changed to a carbon atom, is not affected by the serine 49 to alanine mutation. This is consistent with the concept that the N-5 position is the main site for serine 49-flavin interaction. In the ferredoxin-NADP+ reductase family, the equivalent serine residue, which has been shown to be essential for activity, is hydrogen-bonded to the N-5 of the FAD cofactor. Taken together, these data provide the first experimental support to the hypothesis that the flavin reductase Fre may belong to the ferredoxin-NADP+ reductase family.

The NAD(P)H:flavin oxidoreductase (or flavin reductase) from Escherichia coli, also named Fre, is the prototype of a group of enzymes, the flavin reductases, which are defined by their ability to catalyze the reduction of free flavins, riboflavin, flavin mononucleotide (FMN), or flavin adenine dinucleotide (FAD) by reduced pyridine nucleotides, NADPH or NADH (1, 2). The physiological importance of such enzymes is still unclear, even though there is indirect evidence for their function, at least in prokaryotes, in bioluminescence (3, 4), ferric reduction (5), oxygen reduction (6), antibiotic synthesis (7, 8), and activation of ribonucleotide reductase (1), the enzyme responsible for the biosynthesis of the deoxyribonucleotides. Flavin reductase activities have been detected also in eukaryotic cells (9), one of them is better know as a methemoglobin reductase (10).

Since very little is known as far as the structure and the catalytic mechanism of these enzymes are concerned, the flavin reductase from E. coli has become the focus of recent efforts to unravel its biochemistry. The corresponding fre gene has been cloned, sequenced, and overexpressed (11, 12). This now allows the preparation of large amounts of pure protein, in particular for crystallographic studies. The flavin reductase consists of a single polypeptide chain of 232 amino acids, with a molecular mass of 26,111 Da, and does not contain any prosthetic group. The catalysis of the reaction is made possible through the existence of a site which can probably accommodate both reduced pyridine nucleotide and the flavin substrate (1, 12).

In a previous work (12), we demonstrated, by using a variety of synthetic flavin analogs acting either as substrates or as inhibitors, that recognition of the flavin by the polypeptide chain occurs exclusively through the isoalloxazine ring. The 1'-ribityl side chain is not significantly involved in the flavin-protein interaction. Thiol reagents, iodoacetate and N-ethylmaleimide, were found to inhibit the flavin reductase activity (1, 13). In a previous work, the three cysteine residues of the flavin reductase were mutated into serine residues, but none of these mutations affected activity. Cysteine residues are thus not involved in catalysis and substrate binding, but two of them, cysteine 5 and cysteine 214, were suggested to reside in close proximity to the flavin site (13).

On the basis of sequence homologies, it has been proposed that the flavin reductase could belong to a class of flavoprotein enzymes which includes ferredoxin-NADP$^+$ reductase (FNR),$^1$ cytochrome P-450 reductase, cytochrome b$_6$ reductase, nitrate reductase, phthalate dioxygenase reductase, sulfite reductase and others (14). Four members of this family have been structurally characterized (15–19).

Although the overall sequence similarity is below significance, the flavin reductase contains a sequence of four amino acids, starting from arginine 46 and ending with serine 49, which is present, highly conserved, within the whole FNR

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$^1$ The abbreviations used are: FNR, ferredoxin-NADP$^+$ reductase; Rf, riboflavin.
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FreV.f, FNR family. gous region of other Fre proteins and several members of the FNR family. Fre E.c, E. coli Fre; Fre P.I, P. luminescens Fre (20); FreV.I, V. fischeri Fre (20); FNR, spinach ferredoxin-NADPH reductase (21); PDR, Pseudomonas cepacia phthalate dioxygenase reductase (17); Nr, cytochrome b reductase fragment of corn nitrate reductase (22); b5r, human cytochrome b5 reductase (23); NOs, rat nitric-oxide synthase (24); Sir, E. coli sulfite reductase (25); P450r, human cytochrome P-450 reductase (26).

**Scheme 1.** Alignment of amino acid 46-49 sequence of E. coli flavin reductase Fre protein with the corresponding homologous region of other Fre proteins and several members of the FNR family, Fre E.c, E. coli Fre (11); Fre P.I, P. luminescens Fre (20); Fre V.I, V. fischeri Fre (20); FNR, spinach ferredoxin-NADPH reductase (21); PDR, Pseudomonas cepacia phthalate dioxygenase reductase (17); Nr, cytochrome b reductase fragment of corn nitrate reductase (22); b5r, human cytochrome b5 reductase (23); NOs, rat nitric-oxide synthase (24); Sir, E. coli sulfite reductase (25); P450r, human cytochrome P-450 reductase (26).

For routine plasmid manipulation, E. coli DH5α was used. Overexpression of mutated flavin reductase was done using E. coli LS1312 (fre::kan) (29). E. coli cultures were grown in Luria-Bertani medium (LB) supplemented, when necessary, with the appropriate antibiotics. Site-directed Mutagenesis—DNA manipulations were done as described in Ref. 30. DNA sequencing was done by the dideoxy Sanger method (31). The Kunkel method was used for mutagenesis (32). The 800-nucleotide EcoRI-HindII fragment from pEE1014 (12), containing the fire gene, was cloned into the replicative form of M13mp18 to give M131014. M131014 single strand, isolated from E. coli CJ 236, was used as a template for mutagenesis. The mutated fire genes were completely sequenced and subcloned into the expression vector pf F119EH, as described previously for the wild-type fire gene (12).

Overexpression and Purification of the Mutated Proteins—Plasmid pf F119EH carrying the mutated fire genes were transformed into E. coli LS1312 (fre::kan). Culture and isopropyl-β-D-galactopyranoside inductions were done as reported elsewhere (12). Purification of wild-type and mutated flavin reductase proteins were done using a two-step protocol, phenyl-Sepharose and Superdex 75, as described previously (12).

Western Blot Analyses—E. coli cells containing the different overexpression plasmids were grown in LB medium and isopropyl-β-D-galactopyranoside-induced as described above. 1 ml of culture was pelleted and resuspended in 50 µl of electrophoresis denaturating buffer. Proteins were then separated on 12% polyacrylamide slab gel electrophoresis in the presence of SDS. After transfer on nitrocellulose membrane, revelation of flavin reductase proteins was performed using a 1,000-fold diluted rabbit serum prepared against pure flavin reductase.

**Evidence Assay—Flavin reductase activity was determined at 25°C from the decrease of the absorbance at 340 nm (ε340 = 6.22 mmol−1 cm−1) due to the oxidation of NADPH, using a KONTRON UVIKON 930 spectrophotometer. Under standard conditions, the spectroscopic cuvette contained, in a final volume of 500 µl, 50 mM Tris-HCl, pH 7.5, 0.2 mM NADPH, and 15 µM riboflavin. Kinetic studies were performed by varying the concentration of NADPH and riboflavin. The reaction was initiated by adding 0.4–12 µg of enzyme, depending on the specific activity of the protein. One unit of activity is defined as the amount catalyzing the oxidation of 1 nmol of NADPH per min. Activity in the presence of lumichrome was measured as previously reported (12). Protein concentration was determined by the method of Bradford (33). Circular Dichroism Spectrophotometry—CD spectra were measured at 25°C with a J OBIN YVON CD6 polarimeter. The magnitude of CD spectra was expressed as a molar ellipticity (θ), degrees·cm2·dmol−1, based on the molecular mass of the flavin reductase (26,111 Da).

**RESULTS**

Preparation of Mutant Enzymes with Serine-49 Substitutions for Threonine or Alanine Residues—We have constructed 2 mutant expression plasmids using the degenerated oligonucleotides listed in Table I and the expression system previously reported (12). In order to avoid contamination by the wild-type flavin reductase from the host strain, the two mutant enzymes were expressed in an E. coli LS1312 (fre::kan) strain, a K12 derivative in which the fire gene was insertionally inactivated with a kanamycin cassette (29). Western blots from extracts of overexpressed flavin reductase proteins, using rabbit antiserum raised against flavin reductase, showed that the S49T mutant enzyme was expressed at a level approximately equivalent to the wild-type protein. On the other hand, the expression of the S49A mutant enzyme was significantly decreased (data not shown). Nevertheless, S49T and S49A mutant enzymes were both purified efficiently using phenyl-Sepharose chromatography and gel filtration on Superdex 75 as described previously for the wild-type protein (12). They exhibited the same elution profile as the wild-type protein. Each purified protein gave a single band on SDS-polyacrylamide gel electrophoresis at the same mobility as the wild-type enzyme (data not shown). Purification yielded 1.2 and 0.4 mg of pure S49T and S49A mutant enzymes, respectively, from 1 g of wet E. coli cells.

Circular Dichroism Spectra and Thermal Stability of the Mutant Enzymes—In order to verify that the global structure of the flavin reductase was not altered by the mutation, we com-
pared the CD spectra of wild-type flavin reductase and S49A mutant. As shown in Fig. 1A, the two proteins have similar spectra, with slight differences which could account for small differences in the concentrations of the two protein samples.

The structural integrity of the S49A and S49T mutants was also assessed by comparing their heat stability to that of the wild-type. Diluted flavin reductase (0.26 mg/ml) was incubated at increasing temperature for 10 min, and aliquots were assayed for activity. As shown in Fig. 1B, no difference between mutants S49A, S49T, and wild-type protein could be observed as far as their thermostability is concerned: all proteins were heat-denatured at 46°C.

Steady-state Kinetics—In Table II are shown the kinetic parameters determined for the wild-type and the two serine 49 mutant enzymes. For assaying flavin reductase activity under standard conditions, NADPH was used as the electron donor and riboflavin as the electron acceptor.

The S49T mutation, in which at position 49 the polarity of the side chain, due to the presence of an hydroxyl group, is maintained, had no effect on the $K_m$ values for both riboflavin and NADPH. The $k_{cat}$ value was reduced about 2-fold. Thus, substitution of threonine for serine had only a minor effect on the activity of the flavin reductase.

The S49A enzyme, in which the side chain of residue 49 is now smaller and has lost the hydroxyl group, displayed $K_m$ values for both riboflavin and NADPH about 5-fold larger than the corresponding $K_m$ values measured with the wild-type enzyme. An increase of the same order of magnitude of $K_m$ values for other flavins, FMN or lumiflavin, was also observed (data not shown).

On the other hand, the S49A mutation had a dramatic effect on the $k_{cat}$ value which was reduced 35-fold. As a consequence, the catalytic efficiency of the mutant, expressed in terms of $K_m K_{cat}^2(Rf)$, was greatly decreased to about 0.2% with respect to the wild-type enzyme catalytic efficiency. $K_m(NADPH)$ is the dissociation constant determined for NADPH as described below (Table I).

Dissociation Constants for NADPH—In a previous work (12), we have shown that E. coli flavin reductase exhibits a sequential ordered mechanism, with NADPH binding first and flavin second (Scheme 2). Experimental $K_m$ values for substrates may not reflect the thermodynamic dissociation constant of enzyme-substrate complexes. As a consequence, the effects of the S49A mutation on both $k_{cat}$ and $K_m$ values may be difficult to interpret and thus not illuminate the structural-catalytic role of the residue. We thus found it necessary to determine true $K_d$ values.

A mathematical treatment of the kinetic data, which was initially developed by Dalziel (35) in the case of dehydrogenases with an ordered mechanism, allows us to determine the $K_d$ value for the first substrate, NADPH. The Dalziel formalism applied to the flavin reductase can be described as follows.

The reciprocal initial velocity equation for a two-substrate reaction can be written as:

$$\epsilon = \frac{e}{v} = \frac{d_{\text{NADPH}}}{[\text{NADPH}]} + \frac{d_{\text{flavin}}}{[\text{flavin}]} + \frac{d_{\text{NADPH}}d_{\text{flavin}}}{[\text{NADPH}][\text{flavin}]}$$  \hspace{1cm} (Eq. 1)

where $e$ is the enzyme concentration, $v$ is the initial velocity, $d_{\text{NADPH}}$, $d_{\text{flavin}}$, and $d_{\text{NADPH}}d_{\text{flavin}}$ are partial rates, and $[\text{NADPH}]$ and $[\text{flavin}]$ are the substrate concentrations.

Using velocity constants $k$ numbered as shown in Scheme 2, the $\phi$ constants can be written as:

$$k_{cat} = \frac{1}{d_0} = k_3k_5k_7$$  \hspace{1cm} (Eq. 2)

$$K_{cat}(\text{NADPH}) = \frac{d_{\text{NADPH}}}{d_0} = k_2k_3k_5k_7$$  \hspace{1cm} (Eq. 3)

$$K_m(\text{flavin}) = \frac{d_{\text{flavin}}}{d_0} = k_1k_3k_5k_7$$  \hspace{1cm} (Eq. 4)

$$\phi_{\text{NADPH}}(\text{flavin}) = \frac{d_{\text{NADPH}}d_{\text{flavin}}}{d_0} = \frac{k_1k_3k_5k_7k_9}{k_1k_3k_5k_7}$$  \hspace{1cm} (Eq. 5)

From these equations, it appears that neither the $K_m$ values for NADPH nor flavin even approximate the thermodynamic dissociation constants for binding of NADPH or flavin. How-
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**TABLE II**

| Dissociation constants of mutant and wild-type flavin reductases for NADPH, lumichrome, and 5-deazariboflavin |
|---------------------------------------------------|
| $K_d$ (NADPH) | $K_d$ (lumichrome) | $K_d$ (5-deazariboflavin) |
|-------------|-----------------|-----------------|
| Wild-type   | 61              | 0.5             | 21              |
| S49T        | 16              | 3               | 21              |
| S49A        | 166             | 8.3             | 32              |

A series of such primary plots have been obtained with different concentrations of flavin. Two secondary plots were then constructed, giving the slopes and intercepts of the primary plots against 1/[flavin], respectively. The former gives a straight line from which the slope, $\phi_{NADPH}$, and the intercept, $\phi_0$, can be determined; the second is also a straight line with $\phi_{NADPH}$ as the slope and $\phi_0$ as the intercept. Then, $K_d$ values were calculated as described above. $K_d$(NADPH) values for S49T and S49A mutant enzymes are reported in Table III.

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| Dissociation constants of mutant and wild-type flavin reductases for NADPH, lumichrome, and 5-deazariboflavin |
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| S49T        | 16              | 3               | 21              |
| S49A        | 166             | 8.3             | 32              |

**DISCUSSION**

In the absence of a three-dimensional structure of the flavin reductase, it is difficult to predict which amino acids play an

lumichrome will reflect similar effects on the $K_d$ value for riboflavin.

In the case of an enzyme with an ordered sequential mechanism, a $K_d$ value for an inhibitor, competitive with respect to the second substrate, can be determined from the equation:

$$K_d = K_d(1 + A/K_A)$$

where $K_d$ is the inhibition constant, and $A$ and $K_A$, the concentration and dissociation constant of the first substrate, respectively (36). $K_d$ values for lumichrome have been obtained for the wild-type and mutant flavin reductases (Table III).

**SCHEME 2. Reaction catalyzed by the flavin reductase.**

- Fre + NADP$^+$ + flavin$^\alpha$ $\rightarrow$ Fre*NADP$^+$ + flavin$^\alpha$ + H$^+$
- Fre + NADP$^+$ + flavin$^\alpha$ $\rightarrow$ Fre*NADP$^+$ + flavin$^\alpha$ + H$^+$

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**TABLE II**

**TABLE II**

Kinetic parameters of mutant and wild-type flavin reductases for NADPH oxidation assayed with riboflavin as an electron acceptor

| $k_{cat}$ | $K_m$(NADPH) | $K_m$(Rf)$^a$ | $k_{cat}/K_d$(NADPH) | $K_m$(Rf)$^a$ |
|-----------|---------------|---------------|----------------------|---------------|
| $s^{-1}$  | $\mu M$       | $\mu M$       | %                    | %             |
| Wild-type | 52            | 30            | 2.5                  | 100$^c$       |
| S49T      | 20            | 42            | 2.4                  | 135           |
| S49A      | 1.5           | 160           | 12                   | 0.2           |

$^a$ $K_d$(NADPH) values were reported in Table III.

$^b$ $K_d$(Rf) values were calculated as described above.

$^c$ The value of the catalytic efficiency is 0.34 $s^{-1}$ $\mu M^{-2}$ for the wild-type enzyme.

**TABLE III**

Dissociation constants of mutant and wild-type flavin reductases for NADPH, lumichrome, and 5-deazariboflavin

| Dissociation constants of mutant and wild-type flavin reductases for NADPH, lumichrome, and 5-deazariboflavin |
|---------------------------------------------------|
| $K_d$ (NADPH) | $K_d$ (lumichrome) | $K_d$ (5-deazariboflavin) |
|-------------|-----------------|-----------------|
| Wild-type   | 61              | 0.5             | 21              |
| S49T        | 16              | 3               | 21              |
| S49A        | 166             | 8.3             | 32              |

A series of such primary plots have been obtained with different concentrations of flavin. Two secondary plots were then constructed, giving the slopes and intercepts of the primary plots against 1/[flavin], respectively. The former gives a straight line, from which the slope, $\phi_{NADPH}$, and the intercept, $\phi_0$, can be determined; the second is also a straight line with $\phi_{NADPH}$ as the slope and $\phi_0$ as the intercept. Then, $K_d$ values were calculated as described above. $K_d$(NADPH) values for S49T and S49A mutant enzymes are reported in Table III.

Dissociation Constants for Flavins—As mentioned above, it was not possible to determine the $K_d$ value for flavin, the second substrate. Lumichrome, which has been found to be a competitive inhibitor of the flavin reductase for the flavin site (12), is an analog of the isoalloxazine ring of riboflavin (Scheme 3). This inhibitory effect reflects the importance of the ring in the recognition of the flavin by the polypeptide chain. We thus assume that the $K_d$ value for lumichrome will reflect that for riboflavin and that effects of mutations on the $K_d$ value for lumichrome will reflect similar effects on the $K_d$ value for riboflavin.

In the case of an enzyme with an ordered sequential mechanism, a $K_d$ value for an inhibitor, competitive with respect to the second substrate, can be determined from the equation:

$$K_d = K_d(1 + A/K_A)$$

where $K_d$ is the inhibition constant, and $A$ and $K_A$, the concentration and dissociation constant of the first substrate, respectively (36). $K_d$ values for lumichrome have been obtained for the wild-type and mutant flavin reductases (Table III).

S49A mutation caused only a 2.7-fold increase of the $K_d$(NADPH) value, but a 17-fold increase of the $K_d$(lumichrome) value. The modification of serine 49 residue to a threonine had a weaker effect on $K_d$ (lumichrome), but still increased it 6-fold with respect to the wild-type protein. This strongly suggested that the lateral chain of serine 49 residue is important for the recognition of the isoalloxazine ring of the flavin molecule by the protein and the stabilization of the ternary (flavin reductase-NADPH-flavin) complex. It is interesting to note that a threonine at position 49 provided a slightly better binding of NADPH.

In the ferredoxin-NADP$^+$ reductase from spinach, a serine residue (Ser-96) is connected through hydrogen bonds to N-5 of the isoalloxazine ring (15–16). In order to see whether, in the case of the flavin reductase, a similar connection exists, we used 5-deazariboflavin where N-5 is replaced by a carbon atom (Scheme 3). 5-Deazariboflavin is a substrate of the flavin reductase but exhibited a very low specific activity (0.7% with respect to riboflavin, when assayed with NADPH as the electron donor (data not shown)).

Because of such a very low specific activity, it was possible to use 5-deazariboflavin as a dead-end inhibitor of the flavin reductase. When the enzyme activity was determined as a function of riboflavin concentration in the absence or in the presence of two concentrations of 5-deazariboflavin, double reciprocal plots reflected typical competitive inhibition kinetics (Fig. 2). As described above for lumichrome, $K_d$ values of 5-deazariboflavin for wild-type and serine 49 mutants can be determined (Table III).

Comparison of these values to the $K_d$ values for lumichrome is correct since the ribetyl chain is not involved in the binding of the flavin (12). The $K_d$ value for 5-deazariboflavin was 42-fold larger than that for lumichrome, in the case of the wild-type enzyme. Furthermore, while mutations at serine 49 greatly increased the $K_d$ value for lumichrome (for 0.5 $\mu M$ to 8 $\mu M$), they had almost no effect on the $K_d$ value for 5-deazariboflavin (from 21 to 32 $\mu M$). It thus appears that changing N-5 for a carbon atom in the flavin molecule or removing the OH group of the lateral chain of residue 49 decreased the affinity of the protein for the flavin moiety to a great extent.
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important role in substrate binding and/or catalysis. In our case, we were rather convinced by the recent suggestion that the flavin reductase could be related to a class of flavin-dependent enzymes, the so-called FNR (ferredoxin-NADP⁺ reductase) family, even though the overall amino acid sequence identities within this family are at below the limit of significance. A few residues are conserved in this class of protein and are thus supposed to be required for maintenance of structure or catalytic function. The importance of these residues has been underlined on the basis of structural data available for FNR (15, 16), phthalate dioxygenase reductase (17), nitrate reductase (18), and cytochrome b₅ reductase (19). The structures have shown that these proteins are organized in two domains, an N-terminal one which interacts with the flavin cofactor and a C-terminal one responsible for NAD(P) binding. Sequence alignment with the flavin reductase and structurally characterized FNR family proteins showed conservation of two fingerprint sequences localized in the N- and C-terminal part of the flavin reductase polypeptide. They correspond to the sequences which interact with the isoalloxazine ring of the flavin and to the pyrophosphate bridge of the nicotinamide cofactor (glycine-rich motif, GGTG, positions 110 to 113 in the flavin reductase), respectively. These observations could suggest a similar organization of the flavin reductase in two domains.

The N-terminal fingerprint sequence (Scheme 1) was used as a guide for the site-directed mutagenesis study reported here. The key function of the last residue of this stretch, a serine or a threonine residue, has been underlined on the basis of structural data and more recently on the basis of the dramatic effects of changing serine 96 of FNR to glycine or valine on enzyme activity (27).

In this paper, we show that the serine residue, at position 49 in the case of the flavin reductase, is also an important residue for enzyme activity. The replacement of Ser-49 by an alanine residue resulted in a large decrease of the $k_{cat}$ value, which represented about 2-3% with respect to the wild-type activity, and a 5-fold increase of the $K_m$ values for NADPH and flavin. As a consequence, the catalytic efficiency is 0.2% that of the wild-type enzyme. Circular dichroism spectra and thermal stability of the mutant protein are the same as the wild-type. Thus, a gross structural rearrangement of the protein conformation due to the mutation cannot be the cause of the loss of activity.

Then, it appeared that an important serine residue may be common to the flavin reductase and FNR. Of course, in the absence of a three-dimensional structure, it is difficult to show unambiguously that serine 49 occupies the active center of the flavin reductase the same strategic position as serine 96 in FNR. As can be seen from the crystal structure of FNR (15, 16), Ser-96 uses its hydroxyl side chain to develop hydrogen bonds with the N-5 position of the isoalloxazine ring. We have thus determined the effect of the Ser-49 mutation on real $K_d$ values for NADPH and flavin analogs considered as second substrates of the reaction catalyzed. In fact, $K_d$ values determined for both wild-type and mutant proteins are more reliable than $K_m$ values for evaluating the effect of the mutation on substrate binding (Tables II and III). Our data, in addition to the sequence homology, are consistent with the suggestion that Ser-49 of the flavin reductase is interacting with the N-5 position of the isoalloxazine ring.

(i) The serine 49 to alanine mutation only increased the real $K_d$ value for NADPH to a minor extent. The same small effect on NADPH binding was observed in FNR in the case of the serine 96 to glycine or valine mutation (27).

(ii) The $K_d$ value for a flavin such as lumichrome was increased 17-fold, suggesting that serine 49 in the flavin reductase is involved in the binding of the isoalloxazine ring. The fact that the $K_d$ value for 5-deazariboflavin, in which the N-5 position has been changed to a carbon atom, is not affected by the serine 49 to alanine mutation is consistent with the concept that the N-5 position is the main site for the serine 49-flavin interaction. One can speculate that this interaction might electronically predispose the isoalloxazine ring to reduction. Alternatively, Ser-49 could contribute to the proper orientation of the flavin toward the nicotinamide ring of NAD(P)H in order to optimize hydride transfers. In FNR, it has been shown that the serine to valine mutation, which results in an enzyme with an extremely low activity (0.05% of the activity of the wild-type protein), affected the interaction of the nicotinamide ring with the flavin cofactor (27). With this particular FNR mutant, it was not possible to detect the charge-transfer intermediate,
normally formed during flavin reduction by NADPH, anyhow. Preliminary rapid reaction experiments with the flavin reductase wild-type enzyme clearly show the transient formation of a long wavelength absorbing species, with properties that allow its identification with a charge-transfer complex between enzyme-bound NADPH and riboflavin. That the existence of such an intermediate could be affected by the serine mutation in the flavin reductase will be studied in the future.

Flavin reductases have been incompletely characterized so far, and this is the first report of a mutation of a totally conserved residue resulting in such a dramatic effect on the flavin reductase activity. We suggest that the serine residue plays an important role in the binding of the flavin through an interaction with the N-5 of the isoalloxazine ring. Since this serine residue was selected on the basis of sequence similarity to FNR, our data provide the first experimental support to the hypothesis that the flavin reductase belongs to the FNR family. Also, that a serine to threonine mutation, at position 49 of the flavin reductase, had only a minor effect on the activity of the enzyme is in line with the presence of a threonine instead of a serine at this position in several members of the FNR family, such as nitrate reductase (18) or cytochrome b₅ reductase (23).

What makes flavin reductases apart from the other members of the FNR family is their much lower affinity for flavins which is in the micromolar range while it is in the nanomolar range in the case of FNR for example. In a previous work, we demonstrated that recognition of the flavin by the flavin reductase occurs exclusively through the isoalloxazine ring (12). On the contrary, FNR polypeptide chain makes extensive hydrogen bonds with the ribityl side chain, the pyrophosphate groups, and the adenine moiety of FAD. Amino acid residues of FNR which interact with these parts of FAD were not found to be conserved in flavin reductase proteins. Thus, such additional interactions, which do not exist in the flavin reductase, could explain why the flavin reductase can use the flavin as a substrate instead of as a cofactor. However, it is tempting to speculate that flavin reductases and flavoproteins of the FNR family may have some evolutionary relationship. They may have derived from each other owing to subtle variations of the amino acid residues constituting the flavin binding site.

From a physiological point of view, the differences in terms of flavin affinity between flavin reductases and flavoproteins have important implications. In both cases, the reducing power of the cell is transferred to a flavin moiety. In the case of a flavoprotein, this reducing power is tightly retained within the protein and access to it is highly constrained. As a consequence, the system is able to finely tune the electron acceptor specificity. Such reductases in general are highly specific for a given substrate, ferredoxin, for example, in the case of FNR. On the contrary, in the case of flavin reductases, the reducing power is liberated in solution, and, thus, very little electron acceptor specificity is allowed. The physiological function of flavin reductases is still uncertain. A likely possibility is that they do not have a specific function but rather serve as a general cytosolic electron source. This would be consistent with the fact that flavin reductases have been discovered in many very different contexts: iron metabolism, oxygen metabolism, DNA replication, bioluminescence, and antibiotic synthesis.

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