Journal of Bioenergetics and Biomembranes, Vol. 26, No. 1, 1994

Structure–Function Relations for Ferredoxin Reductase

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Received September 8, 1993; accepted October 5, 1993

Ferredoxin:$NADP^+$ reductase is representative of a large family of flavoenzymes which catalyze the interchange of reducing equivalents between one-electron carriers and the two-electron-carrying nicotinamide dinucleotides. The structure of the enzyme from spinach is known at 1.7 Å resolution and this structure, together with results of chemical modification and site-directed mutagenesis studies, gives insights into features of the structure that are important for function.

KEY WORDS: Ferredoxin reductase; ferredoxin; protein structure; structure–function relations; site-directed mutagenesis.

INTRODUCTION

Ferredoxin:$NADP^+$ oxidoreductase (FNR) is the last enzyme in the electron transport chain of linear photosynthesis and catalyzes the transfer of electrons from two molecules of reduced ferredoxin (Fd) to a single molecule of $NADP^+$ according to the reaction

$$2Fd_{reduced} + NADP^+ + H^+ \rightarrow 2Fd_{oxidized} + NADPH$$

In catalyzing this reaction FNR utilizes a noncovalently bound FAD prosthetic group. Extensive kinetic studies have been carried out to yield the proposed catalytic cycle shown in Fig. 1 (Batie and Kamin, 1984) which involves a ternary FNR-$NADP^+$-ferredoxin complex.

The structure of FNR appears to be the prototype for a two-domain structural motif that occurs in many enzymes which need to transfer electrons from a nicotinamide dinucleotide, one at a time, to a one-electron acceptor. This large family of enzymes is schematically shown in Fig. 2. It can be seen that these proteins combine other domains with an FNR-like module to make a diverse family. Although this review will focus mostly on FNR itself, using structural and biochemical information from all of these systems provides helpful insights.

FNR is ubiquitous among photosynthetic organisms. In higher plants it is found inside chloroplasts in two fractions: one loosely bound and one tightly associated with the outer surface of thylakoid membranes (Matthijs *et al.*, 1986). The different modes of association may regulate FNR activity, as membrane binding has been shown to affect the kinetic parameters of the enzyme (Carrillo and Vallejos, 1983) and changes in the ratio of loosely and tightly bound enzyme correlate with $NADP^+$-photoreducing activity (Ohasi *et al.*, 1992; Nakatani and Shin, 1992). However, there is not yet any structural explanation for membrane association or activity modulation, and these topics will not be discussed in this review. FNR has also been found in nonphotosynthetic tissues such as plant roots, and in these tissues it may produce reduced ferredoxin from NADPH via the reverse reaction (Morigasaki and Wada, 1990).

The three-dimensional structure of spinach FNR has been described at 2.2 Å resolution (Karplus *et al.*, 1991) and this structural information complements and extends information from biochemical results and known amino acid sequences. In this review we will summarize the structural features of FNR and some insights into structure/function relations. Other recent reviews of FNR covering a broader perspective are

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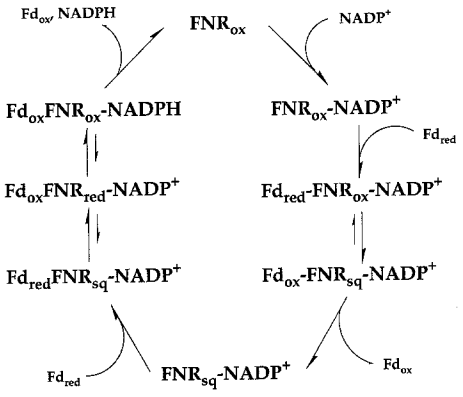

Fig. 1. The catalytic cycle of FNR as reported by Batie and Kamin (1984). FNR_{ox}, oxidized FNR; FNR_{sq}, the one-electron reduced semiquinone form of FNR; FNR_{red}, reduced ferredoxin.

available (Carrillo and Vallejos, 1987; Zanetti and Aliverti, 1991; Knaff and Hirasawa, 1991).

STRUCTURAL KNOWLEDGE

Sequence and Structural Information

Amino acid sequences have been determined for FNR from higher plants, algae, and cyanobacteria. A partial amino-terminal sequence is also known for FNR from spinach and radish roots which shows it to be a unique gene product, and an FNR-like protein sequence has been obtained from rice. Three of these FNRs, those of pea (Ceccarelli *et al.*, 1991), spinach (Aliverti *et al.*, 1990), and *Anabaena* (Fillat *et al.*,

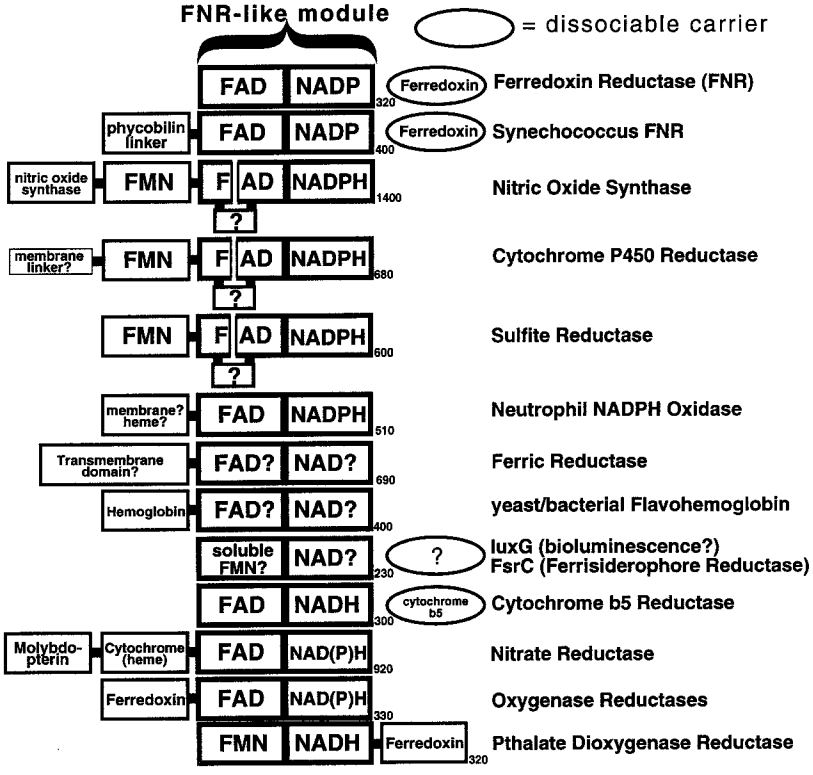

Fig. 2. Domain structure of members of the FNR family of flavoenzymes. Many of these proteins were listed in Andrews *et al.* (1992). References for representative sequences are spinach FNR (Karplus *et al.*, 1984), *Synechococcus* FNR (Schluchter and Bryant, 1992), human nitric oxide synthase (Janssens *et al.*, 1992), human cytochrome P450 reductase (Shephard *et al.*, 1992), *E. coli* sulfite reductase (Ostrowski *et al.*, 1989), human neutrophil NADPH oxidase (Royer-Pokora *et al.*, 1986), *S. cerevisiae* ferric reductase (Dancis *et al.*, 1992), *Candida norvegensis* flavohemoglobin (Iwaasa *et al.*, 1992), *E. coli* ferrisiderophore reductase C (Spyrou *et al.*, 1991), *Photobacterium leiognathi* LuxG protein (Lee *et al.*, 1991), human cytochrome b_5 reductase (Yubisui *et al.*, 1987), tobacco nitrate reductase (Vaucheret *et al.*, 1989), *M. capsulatus* methane monooxygenase C (Stainthorpe *et al.*, 1990), and *Pseudomonas cepacia* phthalate dioxygenase reductase (Liu and Zylstra, 1992). A partial alignment of these sequences appears in Bruns and Karplus (1993).

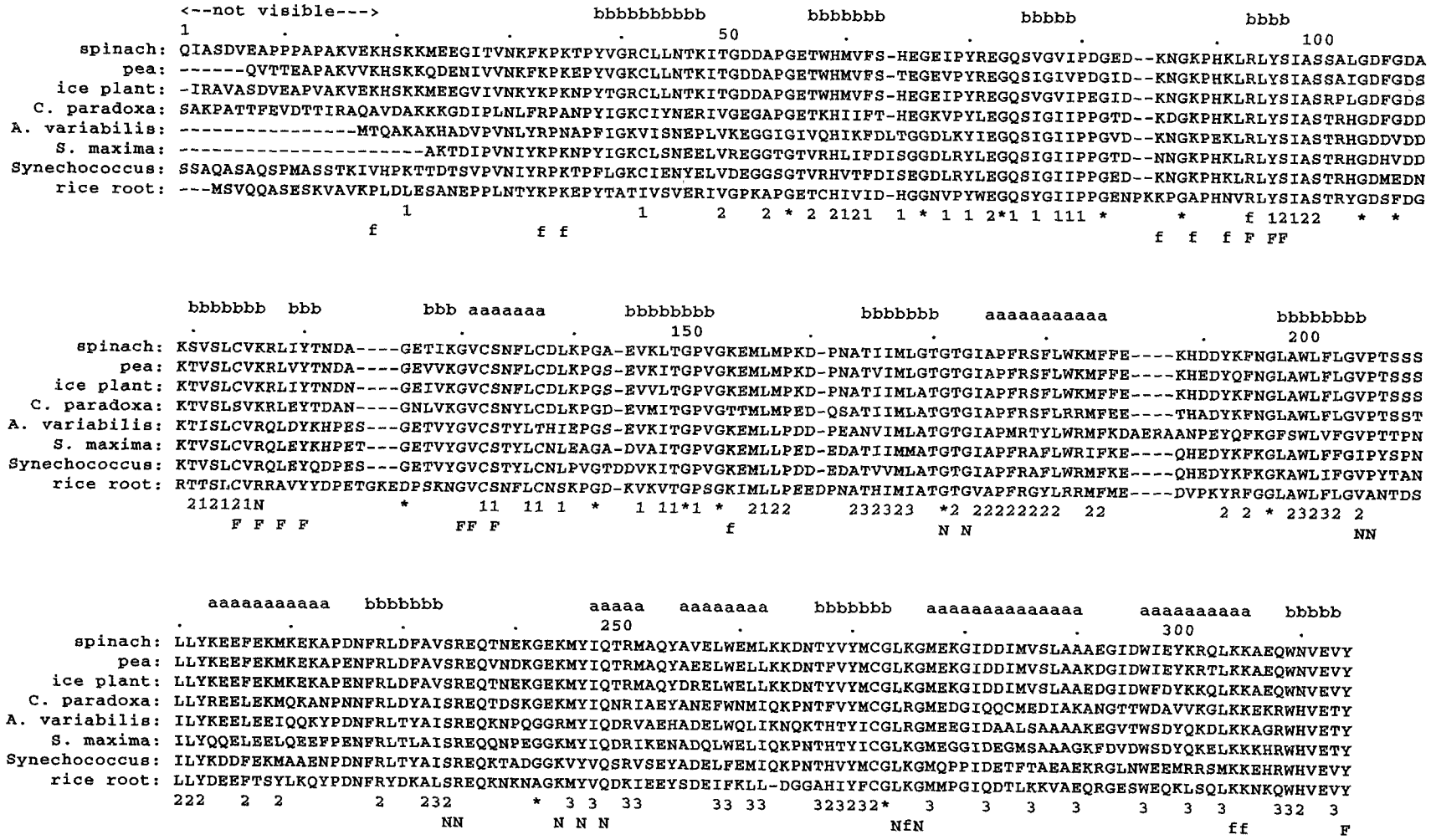

Fig. 3. The known amino acid sequences of FNR from various organisms. The first line summarizes the secondary structure (a: α-helix, b: β-strand). The second line gives residue numbers for the spinach sequence. The central lines give the sequences of spinach (Karplus *et al.*, 1984; Jansen *et al.*, 1988), pea (Newman and Gray, 1988), ice plant (Michalowski *et al.*, 1989), *Cyanophra paradoxa* (Jakowitsch *et al.*, 1993), *Anabaena* PCC 7119 (Fillat *et al.*, 1990), *Spirulina maxima* (Yao *et al.*, 1984), *Synecococcus* (Schluchter and Bryant, 1992), and rice (Aoki and Ida, 1992). *Anabaena* FNR was recently shown to have a large N-terminal extension much like *Synechococcus* (Fillat *et al.*, 1993a). The following lines contain information about residue environment: 1, 2, or 3 indicates residues involved in one of the buried cores; F, N, and f denote FAD binding, NADP binding, and possible ferredoxin binding residues, respectively; * indicates a glycine with special ϕ, ψ angles.

Table I. Percent Identities for FNR from Various Sources

	Spinach	Pea	*M. Cryst.*	*C. para.*	*A. var.*	*S. max.*	*Synec.*	Rice
Spinach	100	87	88	60	50	54	52	48
Pea	—	100	85	61	53	56	53	49
M. crystallinum	—	—	100	60	51	56	50	48
C. paradoxa	—	—	—	100	55	61	48	49
A. variabilis	—	—	—	—	100	69	62	47
S. maxima	—	—	—	—	—	100	71	48
Synechococcus	—	—	—	—	—	—	100	46
Rice root	—	—	—	—	—	—	—	100

1993b), have been cloned and overexpressed to provide a ready source of protein and the possibility of site-directed mutagenesis. The sequences are all shown in Fig. 3, and the pairwise percent identities (Table I) indicate the relative levels of similarity. It is clear from these results that with the exception of the rice protein, the higher plant FNRs are all very similar to one another and much more divergent from the cyanobacterial and algal FNRs. An intriguing discovery was that the *Synechococcus* FNR has a long amino terminal extension (not shown in Fig. 3) that is related to the phycobiliprotein linker polypeptides (Schluchter and Bryant, 1992). Although this extra domain may be important for localization it does not appear to be involved in the enzyme activity, and almost certainly folds independently.

We have tentatively identified the rice FNR-like protein as rice root FNR because its sequence is more similar to the amino terminal sequence reported for spinach root FNR than it is to spinach leaf FNR. In particular the spinach root FNR sequence from residues 14–27 (residue numbers used throughout this review are based on the spinach leaf FNR sequence) is VAVSPLELxDAKEP (Morigasaki and Wada, 1990). This stretch has one identity with spinach leaf FNR and nine identities with the FNR-like protein from rice.

The most divergent pair of sequences shown has an identity level of 46%, which is high enough to suggest that absolute conservation of residues among these sequences is not a reliable indicator of importance. However, in most of the structural description given below, we will refer liberally to the aligned sequences and the observed conservation patterns, because this information still gives valuable insight into the generalizability of features observed in the spinach FNR structure.

Only one crystal structure of FNR, that from spinach leaves, has been reported in the literature (Karplus *et al.*, 1991). It was originally reported at 2.2 Å resolution and has recently been refined at 1.7 Å resolution. However, crystals have also been reported for FNR from *Anabaena* sp. PCC 7119

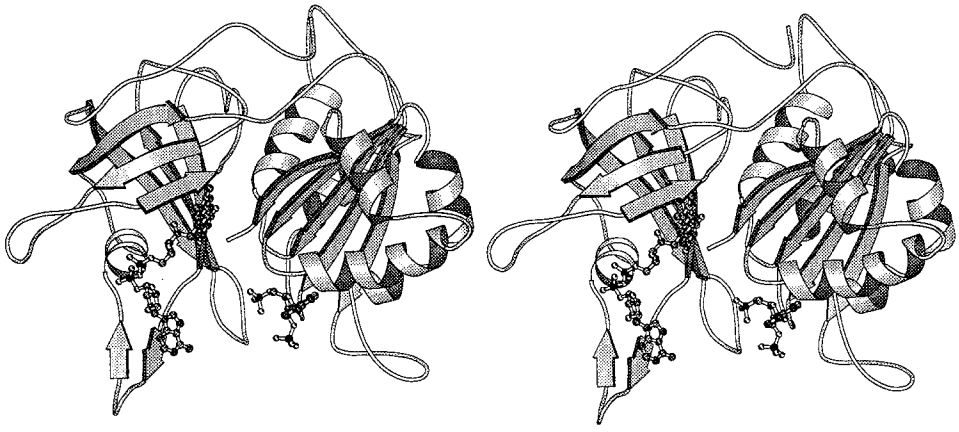

Fig. 4. Stereo view of a ribbon diagram of spinach FNR. β-strands are shown as arrows and α-helices as spirals. Ball and stick models are shown for the FAD and 2′-phospho-5′-AMP.

(Serre *et al.*, 1991), and that structure recently has been solved and refined at near 2 Å resolution (Serre *et al.*, 1993).

Overview of the Three-Dimensional Structure

A stereo ribbon diagram of spinach FNR shows that the protein is made up of two domains (Fig. 4). The first 19 residues of spinach FNR are not visible in the electron density maps and are therefore probably disordered. The visible structure begins at residue 20 and residues 20 through 162 form the first domain, which is a six-stranded antiparallel β-barrel having a peripheral two-stranded antiparallel hairpin leading into the single eight-residue long α-helix. The hairpin and helix bind the adenosine-diphosphate portion of FAD. Residues 20 through 27 and 150 through 161 adopt an extended conformation and are involved in the interface between the FAD-binding domain and the second major structural domain, which is responsible for NADP binding. This region is well conserved among the known FNR sequences and includes a conserved *cis*-proline at residue 150. The NADP-binding domain consists of a central five-stranded parallel β-sheet with six associated α-helices and includes residues 162 through 314. In addition to the residues at the amino terminus, mobile regions of the structure include surface loops near residues 52, 121, and 240. The seven positions which contain gaps or insertions are easily accommodated between regions of secondary structure (Fig. 3). The five Cys residues in spinach FNR are all confirmed to be present as sulfhydryls, as opposed to disulfide bridges (Yao *et al.*, 1985).

Residues Important for Structural Integrity

One class of structurally important residues which is conserved is those which are buried in the hydrophobic core of FNR. The two structural domains and the interface between them enclose three distinct pockets of side chains which are largely hydrophobic and which we will call buried clusters (Fig. 5). One cluster is in the interior of the anti-parallel β-barrel (cluster 1), and two are associated with the parallel β-sheet (one on each side; clusters 2 and 3). Cluster 2 also includes the residues from both domains which make up the interface between the two domains. This intimate association between the two domains suggests that their folding may not be completely independent. Interestingly, cluster 3 is very loosely packed, although it is unknown if this has a functional role. The 103 residues taking part in these clusters are noted in Fig. 3.

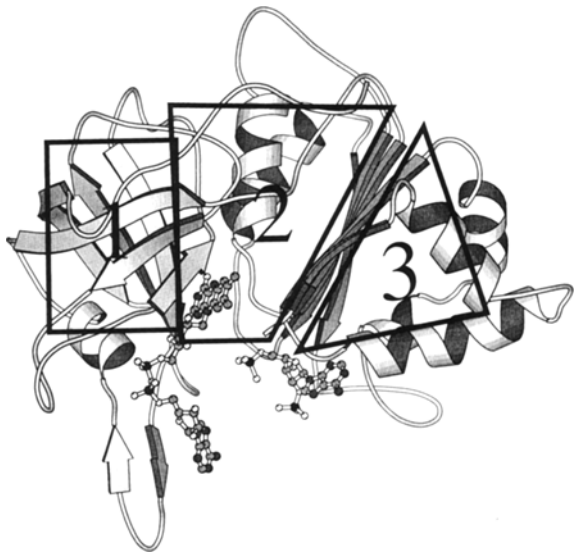

Fig. 5. The buried cores of FNR. The enclosed areas labelled 1, 2, and 3 designate the regions of the three main clusters of buried residues contributing to the folding of FNR.

Other structurally important conserved residues include the *cis*-proline at position 150 and a number of residues which stabilize turn conformations. These include glycines which adopt main-chain dihedral angles that are only allowed for glycines (see Fig. 3), as well as some serine and aspartic acid residues whose side chains make hydrogen bonds to the main-chain atoms of neighboring residues.

Residues Involved in FAD Binding

FAD is bound outside of the antiparallel β-barrel which makes up the core of the FAD binding domain (Fig. 4). The riboflavin is deeply nestled into a pocket formed by strands 4 and 5 of the β-barrel. Tyr-95, Ser-96, and Tyr-314 cover the front and back sides of the flavin, and the pyrophosphate is anchored by Arg-93 and peptide amides from residues Gly-130 and Ser-133 located in the first turn of the α-helix in this domain. Ser-133 also makes a side-chain hydrogen bond to the phosphoryl group. The adenine portion of FAD is bound by only van der Waals contact with the β-hairpin at residues 116–128. Residues that are directly involved in FAD binding are noted in Fig. 2. All of these are absolutely conserved among the FNR sequences known except for Cys-114, which is a serine

in *C. paradoxa* FNR and for which only the main chain is involved in FNR binding.

Residues Involved in NADP Binding

$NADP^+$ must bind to FNR with its nicotinamide ring close to the flavin to allow hydride transfer to occur. Crystallographic analyses have not yet succeeded in showing how the nicotinamide portion of FNR binds. However, a fragment of $NADP^+$, 2′-phospho-5′-AMP, which is a competitive inhibitor with $K_I = 2\mu M$, has been bound in the crystal and reveals the binding mode of the 2′-phospho-AMP half of $NADP^+$ (Karplus *et al.*, 1991). This inhibitor (and presumably $NADP^+$) binds at the C-terminal end of the central β-sheet in the $NADP^+$-binding domain (Fig. 4). The adenine is sandwiched between Tyr-246 and Leu-274 and the 2′-phosphate is specifically recognized by Ser-234, Arg-235, Tyr-246, and possibly Lys-244. The 5′-phosphate group of 2′-5′ ADP interacts with Lys-116 from the FAD domain and approaches the amino terminus of the first α-helix in the NADP binding domain.

Although no structural data are available to explain how the nicotinamide monophosphate portion of $NADP^+$ binds, it is likely that its binding involves a conformational change in FNR. At a minimum this would involve the displacement of Tyr-314, which is stacked on the *re*-face of the flavin, by the nicotinamide of $NADP^+$ (Karplus *et al.*, 1991). As discussed below, biochemical studies imply that a conformational change occurs upon $NADP^+$ binding. Again, residues located in the NADP binding site are noted in Fig. 3, and as was seen for FAD-binding residues, all but one, Pro-205, are absolutely conserved among FNRs.

Residues Involved in Ferredoxin Binding

Because no crystallographic data are available for an FNR:ferredoxin complex, it is not possible based on direct observation to list residues involved in this interface. However, on one face of FNR the dimethylbenzyl portion of the flavin is exposed, and it is likely that ferredoxin binds here with its iron-sulfur cluster approaching the C7a and C8a atoms of the flavin (Karplus *et al.*, 1991). Recently the structure of a distantly related relative of FNR, phthalate dioxygenase reductase, has been solved (Correll *et al.*, 1992). This protein has an FNR-like pair of domains, covalently attached to a ferredoxin-like iron-sulfur domain (Fig. 2), and shows that the iron-sulfur cluster is located very close to the C7a and C8a methyl groups of the flavin. Biochemical data support this docking site and give additional insight into specifics of the interaction as is further discussed below.

CHEMICAL MODIFICATION AND MUTAGENESIS

FNR was first isolated from spinach over 35 years ago, and the spinach enzyme has been the subject of a number of chemical modification studies and recent mutagenesis studies aimed at elucidating structure–function relations. More recently, FNR from *Anabaena* PCC 7119 has also been extensively characterized. We will focus on studies which identified individual residues and not those which only succeeded in implicating a certain amino acid type. These studies are consistent with the crystal structure, and provide important complementary information.

Probes of the FAD Binding Site

Because FAD is very tightly bound to the enzyme, chemical modification studies have not been very useful probes of its binding site. There are reports of a thiol group involved in FAD binding which is sensitive to mercurials (Zanetti and Forti, 1969; Kierns and Wang, 1972). The mutagenesis studies in which each of the Cys residues was individually converted to Ser showed that Cys-272 is partly responsible for the sensitivity to mercurials (Aliverti *et al.*, 1993a). The three-dimensional structure shows Cys-272 to be well buried in the active site near the flavin of FAD. Also, Zanetti *et al.* (1983) have successfully made apo-FNR (FAD-free) and reconstituted it with a variety of modified flavins. These studies gave results which were completely consistent with the crystal structure.

Probes of the NADP-Binding Site

Chemical modification studies on spinach FNR have located two specific residues which can be modified to cause a decrease in enzyme activity and which can be protected from modification by the binding of NADPH. The first of these, Lys-116, was identified through modification first by dansyl chloride (Cidaria *et al.*, 1985), and later by *N*-ethylmaleimide (Aliverti *et al.*, 1991a). The observation that *N*-ethyl-

Table II. Enzymatic Activities of Site-Directed Mutants of Spinach FNR[a]

Enzyme form	cyt *c* reductase activity (%)	Dye reduction	
		k_{cat} (%)	$K_m^{NADP^+}$ (μM)
Wild type	100	100	50
K116Q	2	10	2330
K244Q	65	195	710
C114S	90	nd	nd
C132S	31	190	40
C137S	87	nd	nd
C272S	14	15	140
Wild type	100	100	14
Y314W	45	46	11
Y314F	49	49	12
Y314S	4	4	9
Y314G	0.4	0.3	8
Y314Δ	0.1	0.1	11

[a] Data for the Lys and Cys mutants were taken from Aliverti *et al.* (1991b, 1993a) where the iodonitrophenyl tetrazolium dye was used; data for Tyr mutants were taken from Orellano *et al.* (1993) where the dye potassium ferricyanide was used.

maleimide modifies a lysine residue rather than a cysteine residue emphasizes the importance of characterizing the products of chemical modification studies. The second residue identified by chemical modification is Lys-244. It was labelled by the 2′,3′-dialdehyde derivative of $NADP^+$ which is an affinity reagent for FNR (Chan *et al.*, 1985). To confirm their roles in NADP binding, both of these residues have been mutated to glutamines by Aliverti *et al.* (1991b), and some properties of the mutants are summarized in Table II. Also, a recent study on *Anabaena* FNR using phenylglyoxal modification implicated two arginine residues, the equivalent of Arg 235 and Lys 244 in spinach FNR, as being part of the NADP binding site (Medina *et al.*, 1992).

Again, these results are completely consistent with the crystal structure. In the crystal complex with 2′-phospho-5′-AMP, Lys 116 extends from the FAD domain to make a hydrogen bond to the 5′ phosphate, but it is not clear with which part of the pyrophosphate it will interact within a productive $NADP^+$ complex. Lys-244 is close to the 2′-phosphoryl group of 2′-phospho-5′-AMP, but its side chain is not very well ordered, suggesting that this interaction is not crucial to NADP binding. However, the crystals are at pH 4.5, and thus the 2′-phosphoryl group may not have the full -2 charge it would carry at neutral pH. In contrast, the three other side chains seen to bind the 2′-phosphoryl group, Ser-234, Arg-235 (the residue implicated in *Anabaena* FNR), and Tyr-246, appear much more well fixed. Perhaps Lys-244 is mainly present to provide a net electrostatic attraction to the negatively charged substrate and might not make crucial interactions. The involvement of the polypeptide chain around Lys-244 and Tyr-246 in $NADP^+$ binding is also implied by limited proteolysis studies which showed that 1 mM $NADP^+$ could protect FNR from trypsin cleavage between residues 235 and 250 (Gadda *et al.*, 1990).

Mutagenesis results are also compatible with the structural picture (Table II). Lys-116 is seen to be important for NADP binding, as the K_m for $NADP^+$ of the K116Q mutant is increased 46-fold over wild type (Aliverti *et al.*, 1991b). This mutant also has a 10-fold decrease in the k_{cat} for dye reduction by NADPH (which is known to reflect the rate of enzyme reduction by NADPH; Massey *et al.*, 1970), suggesting that the modification of Lys-116 may affect the geometry of the nicotinamide placement. Lys-244 may be less crucial than Lys 116, as the K_m of K244Q is increased only 14-fold over wild type and its k_{cat} actually increases by a factor of 2.

Probes of the Ferredoxin Binding Site

The binding of spinach FNR to ferredoxin is quite sensitive to ionic strength and is thus thought to be largely electrostatic in nature (Foust *et al.*, 1969; Carillo and Vallejos, 1987). Since the amino acid sequence of ferredoxin has a large number of conserved acidic residues, it is thought that the complex may largely be governed by positive groups on FNR interacting with negative groups on ferredoxin. The interaction is somewhat nonspecific as FNR will form functional complexes with a variety of single-electron carriers from many species (Foust *et al.*, 1969). Recent characterizations of FNR:ferredoxin and FNR:flavodoxin complexes from *Anabaena* PCC 7119 has shown them to be less sensitive to ionic strength (Walker *et al.*, 1991).

Based on the notion that positive residues on FNR will interact with negative residues on ferredoxin, a number of modification studies have been done. Using two different reagents to modify lysine residues, Jelasarov *et al.* (1993) have identified four lysine residues on spinach FNR which are protected from modification by the binding of ferredoxin. One reagent, the *N*-hydroxysuccinimidyl ester of biotin, identified residues 18 and 153, and a fluorescent

isothiocyanate derivative identified residues 33, 35, and 153. Also, in separate studies of phenylglyoxal and pyridoxal phosphate modified *Anabaena* FNR, Medina *et al.* (1992a,b) found that modification of the equivalents of Arg-93 and Lys-305 impaired ferredoxin binding. Lys-53, which is the equivalent of Pro-69 in spinach FNR, was also modified, but this residue is not so well conserved and is structurally more distant from the other implicated residues (Serre *et al.*, 1993). Another study which has implicated specific FNR residues was reported by Zanetti *et al.* (1988). They showed that crosslinking of the FNR:ferredoxin complex linked either Lys-85 or Lys-88 of spinach FNR with one or more of the glutamate residues at positions 92, 93, and 94 of spinach ferredoxin. It should be noted that such crosslinking studies are expected to label residues which are accessible to the solvent and thus at the periphery of the binding region. In contrast, the chemical modification studies will tend to identify residues which are buried in the protein:protein interface.

Forms of spinach FNR which are missing residues at the amino terminus either as a result of the *E. coli* expression system (Aliverti *et al.*, 1990) or as a result of limited proteolysis (Gadda *et al.*, 1990) give additional insight. FNR lacking residues 1–21 is fully active in ferredoxin-dependent enzyme assays, while FNR which starts at residue 29 is about 40% active, and enzyme that starts at residue 33 or 36 is inactive. These truncated enzymes are apparently normally folded because NADPH-dependent diaphorase activity is normal. These results are consistent with chemical modification studies (Jelasarov *et al.*, 1993) which imply the involvement of Lys-33 and 35 in ferredoxin binding.

All of the implicated residues are on the large concave surface of FNR which contains the exposed edge of the dimethylbenzyl ring of the flavin. A stereo view of this surface of FNR with the implicated residues and some other residues which may be involved is shown in Fig. 6. These residues are also noted in Fig. 3. Most of the implicated residues are well conserved, but only the positions of Lys-33, Lys-305, and Arg-93 have positively charged side chains in all known FNR sequences (Fig. 3). Arg-93 is a special case because it is intimately involved in FAD binding and is conserved for that reason. Other conserved basic residues on this surface of FNR include Lys-275 and Lys-304.

Electrostatics calculations using the spinach FNR structure have shown that in addition to specific interactions which may be important for ferredoxin binding, there is a general excess of positive change on this surface of FNR which may help to attract and orient an incoming ferredoxin molecule (De Pascalis *et al.*, 1993). Two strong lobes of the positive electrostatic field are centered on the clusters of lysine residues at 33, 35, 85, 88, and 91 and at 153, 304, and 305.

Using a model for spinach ferredoxin based on the structure of *Aphanothece sacrum* ferredoxin (Tsukihara *et al.*, 1990), De Pascalis *et al.* (1993) showed that the electrostatic field of ferredoxin complements that of FNR. The ferredoxin field has a large negative lobe

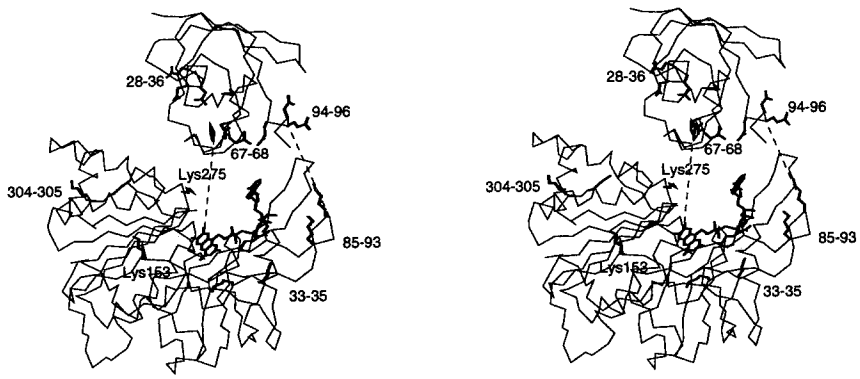

Fig. 6. Stereo view illustrating the residues implicated in ferredoxin binding and a plausible orientation for the interaction. For clarity, the ferredoxin molecule is translated about 10 Å above FNR. Dashed lines roughly designate regions that may come together upon binding. Side chains are shown for residues Lys-33, Lys-35, Lys-85, Lys-88, Lys-91, Arg-93, Lys-153, Lys-275, Lys-304, and Lys-305 from FNR and residues Asp-28, Glu-31, Glu-32, Asp-36, Asp-67, Asp-68, Glu-94, Glu-95 and Asp-96 from *Apanothece sacrum* ferredoxin (subtract 2 for equivalent spinach numbers). The FAD of FNR and the Fe_2-S_2 center of ferredoxin are also shown.

centered on residues Asp-65, Asp-66, Glu-92, Glu-93, and Glu-94, and a smaller negative lobe centered on residues Asp-26, Glu-29, Glu-30, and Asp-34. Acidic residues in these areas of ferredoxin have been implicated in FNR recognition (De Pascalis *et al.*, 1993; Zanetti *et al.*, 1988; Viera *et al.*, 1986). A model of how ferredoxin might dock onto FNR which is consistent with the experimental results is shown in Fig. 6.

As mentioned above, the enzyme phthalate dioxygenase reductase (PDR) contains a ferredoxin-like domain on the same chain as an FNR-like module (see Fig. 2) and shows how they interact. A hypothetical FNR:ferredoxin complex based on that structure is quite similar to the docking mode shown in Fig. 6, but has the ferredoxin molecule rotated nearly 130° around a vertical axis (Correll *et al.*, 1993). In this way very similar faces of FNR and ferredoxin interact, but different groups are brought together. The PDR-analogous rotation of ferredoxin moves residues 92–94 of ferredoxin more than > 25 Å from Lys-85 and 88 of FNR and thus is not consistent with the crosslinking result.

INSIGHTS INTO CATALYSIS

As seen in Fig. 1, the catalytic cycle involves two successive one-electron reductions of the flavin in FNR, and then a hydride transfer to produce NADPH. The electron transfer from ferredoxin to FNR is known to be very rapid (Bhattacharyya *et al.*, 1986; Walker *et al.*, 1991). Given the redox potential difference between ferredoxin and FNR, it is likely that no special catalytic residues are needed, just the close association of the two redox centers upon binding. The fully reduced structure of FNR is known at 2 Å resolution and shows only minor changes from oxidized FNR. The most significant changes are the association of a water molecule at N1 of the flavin and 0.2 Å movement of Ser-96-Oγ toward N5 of the flavin (Bruns and Karplus, unpublished).

A stereo view of the active site region is shown in Fig. 7. Among the residues surrounding the flavin, three are especially well conserved in virtually all known proteins with FNR-like modules (Fig. 2), and are thus likely to be very important in function. These are Ser-96 (conserved as Ser or Thr), Cys-272 (conserved as Cys), and Tyr-314 (conserved as Tyr, Phe or Trp). Interestingly, Pro-176, which contacts Cys-272, and Gly-73 which contacts Pro-176, are also very well conserved. This suggests that the exact positioning of atoms in this region is important.

Karplus *et al.* (1991) postulated that nicotinamide will bind stacked on the flavin ring in a manner similar to that seen for glutathione reductase. This position is occupied by Tyr-314 in the unliganded structure (Fig. 7), which would have to be displaced. Mutagenesis of the equivalent of Tyr-314 in pea FNR has been carried out (Orellano *et al.*, 1993), and the results are given in Table II. The activities of Y314F and Y314W mutants are decreased only by a factor of 2, while Y314S, Y314G, and a deletion mutant showed 25- to 1000-fold activity losses. It thus appears that Tyr-314 may not simply be displaced by nicotinamide, but may play a more active role in catalysis. One possibility is that when it is displaced by nicotinamide, it still plays an important steric role to help orient and fix the nicotinamide in its proper position. Such a role has also been postulated for an

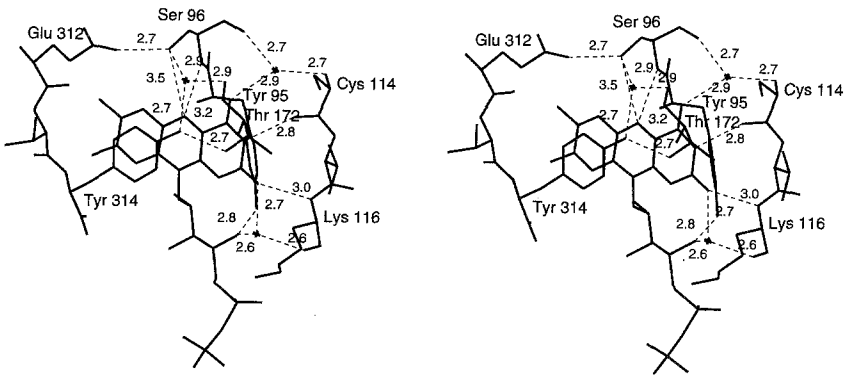

Fig. 7. Stereo view of the environment of the flavin. Hydrogen-bonding interactions are indicated by dashed lines and distances (in Å) are given.

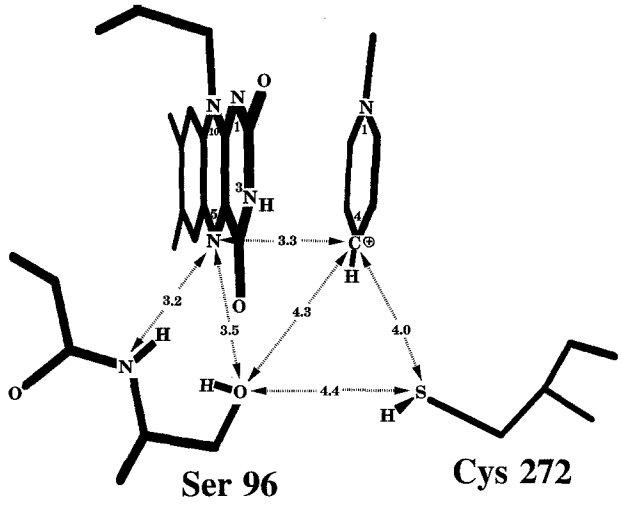

Fig. 8. Postulated nicotinamide binding site (Karplus, 1990).

active-site tyrosine in glutathione reductase (Karplus and Schulz, 1989). This postulated nicotinamide site is shown in Fig. 8 and intimately involves the three well-conserved active site residues: Tyr-314 is displaced as discussed above, and Ser-96 and Cys-272 end up as the closest atoms to N5 of the flavin and C4 of the nicotinamide, the two atoms involved in hydride transfer. Possible catalytic roles suggested for these residues are: (1) the Ser-96 peptide hydrogen bond to N5 could cause the N5 proton present in the reduced state to be pushed out of the flavin plane toward nicotinamide; (2) the Ser-96 hydroxyl could accept a hydrogen bond to help stabilize the reduced flavin and, being located part way between N5 and C4, it is well positioned to affect the transition state of hydride transfer; and (3) Cys-272 could provide a steric aid to the positioning of the nicotinamide, and the sulfur could accept a hydrogen bond from the oxidized nicotinamide to make it more ready to accept a hydride.

A C272S mutant showed a 16-fold decrease in the rate of hydride transfer, consistent with it having a supporting, but not crucial, role in catalysis (Aliverti *et al.*, 1993a). A S96V mutant, on the other hand, had $< 0.2\%$ activity, consistent with a more important role for Ser-96 (Aliverti *et al.*, 1993b).

OUTLOOK

With three species of FNR cloned and over-expressed and a second structure of FNR, that from *Anabaena* soon to be published, we can expect rapid progress to be made in structure–function studies of FNR. Mutagenesis studies will be particularly helpful in defining the specific interactions between FNR and ferredoxin even in the absence of a crystal structure. This improved understanding of FNR activity *in vitro* will then hopefully feed back to improve our understanding of the details of the function and regulation of FNR in its physiological setting.

ACKNOWLEDGMENTS

CMB received support from NIH grant 5T32GM07273. This work was supported in part by NSF grant MCB 9112699. We thank Giuliana Zanetti for helpful discussions.

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