

Review

Interaction of ferredoxin–NADP⁺ reductase with its substrates: optimal interaction for efficient electron transfer

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Abstract

Electron transfer (ET) reactions in systems involving proteins require an oriented interaction between electron donor and acceptor in order to accommodate their respective redox centres in optimal orientation for efficient ET. Such type of reactions are critical for the maintenance of the physiological functions of living organisms, since they are implicated in vital actions, as is, for example, in the photosynthetic ET chain that leads to NADPH reduction. In this particular case, a small redox protein ET chain is responsible for ET from Photosystem I (PS I) to NADP⁺. In this system the enzyme responsible for NADP⁺ reduction is ferredoxin–NADP⁺ reductase (FNR), a FAD-containing NADP⁺ dependent reductase. In order to produce such reduction, this enzyme receives electrons from a [2Fe–2S] plant-type ferredoxin (Fd), which is previously reduced by PS I. Moreover, in the case of some algae and cyanobacteria, an FMN-dependent protein, flavodoxin (Fld), has been shown to replace Fd in this function. The processes of interaction and ET between FNR and all of its substrates involved in the photosynthetic ET chain, namely Fd, Fld and NADP⁺/H have been extensively investigated in recent years using a large number of techniques, including the introduction of site-specific mutations in combination with kinetic and structural studies of the produced mutants. The present manuscript summarises the information so far reported for an efficient interaction between FNR and its substrates, compares such information with that revealed by other systems for which the FNR structure is a prototype and, finally, discusses the implications of the processes of association in ET between FNR and its substrates.

Abbreviations: 2'-P-AMP – 2'-phospho-AMP portion of NADP⁺/H; CPR – cytochrome P450 reductase; dRf – 5-deazariboflavin; ET – electron transfer; Fd – ferredoxin; Fd_{ox} – Fd in the oxidised state; Fd_{rd} – Fd in the reduced state; Fld – flavodoxin; Fld_{ox} – Fld in the oxidised state; Fld_{rd} – Fld in the reduced state; FNR – ferredoxin–NADP⁺ reductase; FNR_{ox} – FNR in the oxidised state; *k*_{ap} – apparent observed rate constant; *k*_{et} – ET first-order rate constant; *K*_d – complex dissociation constant; NMN – nicotinamide mononucleotide portion of NADP⁺/H and NAD⁺/H; PS I – Photosystem I; WT – wild-type

The catalytic reaction of ferredoxin–NADP⁺ reductase

The energy that the living organisms use to perform their physiological functions comes, in the long term, from oxidation–reduction reactions that are coupled to the production of the energy-rich molecule of ATP. In

plants, as in other photosynthetic organisms, the light energy is used to extract electrons from the oxygen that is present in water to reduce NADP⁺. On the contrary, heterotrophic organisms use reduced compounds taken up as nutrients to generate the reduced enzyme cofactors NAD(P)H (a pyridine nucleotide) and the flavin nucleotides FMNH₂ or FADH₂. ATP is formed

during these processes by taking up part of the energy that is liberated in the electron transfer (ET) reactions.

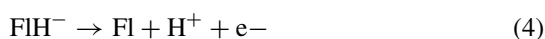
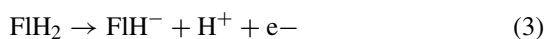
In a similar type of reactions, but proceeding in the opposite direction, reduced pyridine nucleotides NADH or NADPH are used up by all organisms as electron donors for the synthesis of a number of compounds that are continuously produced by the cellular machinery. In many of these oxidation–reduction reactions one of the substrates (SH_2 in reaction (1)) transfers necessarily two electrons while the other (A) is only able to accommodate one electron. The correct stoichiometry of the reaction is assured by the participation of one two-electrons substrate (S) and two one-electron substrates (A), as indicated in reaction (1).



Since, in general, the enzyme can only bind one molecule of substrate A at a time, it has to accommodate transitorily the two electrons before it gives them up one by one. This function is perfectly fulfilled by FMN or FAD, the flavin cofactors that are present in flavoproteins, since the oxidised form of both molecules produces the hydroquinone by accepting two electrons in a single reaction (reaction (2)).



Once it is fully reduced, the electrons can be taken up one by one forming the intermediate species named semiquinone (reaction (3)). This is a radical species that is unstable when the flavin is free in solution but it can be stabilised if the cofactor is inserted into a protein environment. In a second reaction the electron present in the semiquinone is removed yielding the oxidised species (reaction (4)).



Therefore, the flavin cofactor plays a unique role in redox reactions by splitting a two-electrons reaction into two one-electron reactions or *vice versa*. This is the case of those reactions in which electrons have to be transferred from (or to) NAD(P)H to (or from) ferredoxin or flavodoxin.

One of the best characterised enzymes of this type is ferredoxin-NADP⁺ reductase (FNR, EC, 1.18.1.2) that is present in photosynthetic organisms. The spinach enzyme was isolated originally by Avron and Jagendorf (1956, 1957) and many of its relevant features were already defined: it is a weakly membrane-bound enzyme that remains associated to the fraction

of ‘grana’ but it is easily made soluble; it requires ferredoxin to produce NADPH in the presence of illuminated chloroplast; it is a flavoprotein and, finally, it was even crystallised (Shin et al. 1963). The enzyme from *Anabaena* appeared to be similar to spinach FNR, the main difference with this material being the difficulty to remove the accessory pigments characteristic of cyanobacteria, the phycobilins, an issue that was not reached satisfactorily in that early report (Susor and Krogman 1966). Such protein-bound pigments can now be removed by using affinity chromatography techniques such as Cibacron-blue Sepharose columns (Pueyo and Gómez-Moreno 1991).

FNR is not an enzyme specific for photosynthesis. It is also frequent to find flavin-containing reductases in reactions that go in the opposite direction to that of FNR, that is, using NAD(P)H to reduce ferredoxin (or flavodoxin) that are the electron donors in reactions in which the reduction of a compound is required. These reactions include those taking place in bacteria for the reductive activation of enzymes that participate in anaerobic metabolism in *Escherichia coli* (Jung et al. 1999) and those involved in the response to oxidative stress as occurs in *E. coli* (Liocher et al. 1994) or in *Azotobacter vinelandii*, where a NADPH–ferredoxin (flavodoxin) reductase is responsible for removing free radicals generated during the metabolism (Jung et al. 1999). Enzymes that use NAD(P)H to reduce a ferredoxin-type ET protein are also involved in the hydroxylation of phthalate that is used as the carbon source for *Pseudomonas cepacia* (Correll et al. 1992).

It has been stated above that FNR is able to act in both directions, either from Fd_{rd} to NADP^+ or from NADPH towards Fd_{ox} . This is possible because the oxidation–reduction potentials of the members involved in the reaction are in the same range of value (E'_0 of *Anabaena* Fd is -420 mV and that of NADP^+ -320 mV) (Pueyo et al. 1991) yielding free energy values that are close to zero in both cases. This difference in redox potential still explain that the reaction goes more easily towards the production of NADPH, as occurs in photosynthesis, where this reaction is required in high yields, than in the opposite direction, in which case the reaction is used to produce compounds required in lower amounts.

One special feature of reactions in which FNR participates is that the enzyme has to interact with a substrate that is a bulky protein. This requires that the reductase present a portion of its surface that is compatible with the formation of a transient com-

plex between the two proteins. Once the complex is formed the reaction takes place rapidly and without side reactions in which electrons could be released out of the enzyme and produce unwanted by-products. The molecular requirements for this reaction are not fully understood at this moment but can be envisaged as follows: the surfaces of both proteins have to be complementary; weak interactions among the proteins have to be established so that the complex remains stable the necessary time for the reaction to take place; and finally, the cofactors present in the proteins that exchange electrons, are close enough in the complex and with the adequate geometry.

Structural features of FNR

Spinach FNR three-dimensional structure was determined at 2.6 Å by Karplus in 1991 (Karplus et al. 1991) and it was described as the prototype of a family of proteins that play different biochemical roles but that bear similar structures. The structure of *Anabaena* PCC7119 FNR was also described five years later and showed to be similar to the spinach enzyme. The main characteristic of the protein is that it shows two domains: the FAD-binding domain in which the cofactor is accommodated and that includes residues 1–138, and the NADP⁺-binding domain which is responsible for the binding of the nucleotide substrate and that includes residues 139–303. Six antiparallel β -strands arranged in two perpendicular β -sheets constitute the FAD-binding domain. On top of this scaffold there is a short α -helix and a long loop that is maintained by a small two-stranded antiparallel β -sheet. The NADP⁺-binding domain consists of a core of five parallel β -strands surrounded by seven α -helices that is a variant of the Rossmann fold characteristic of dinucleotide binding enzymes (Serre et al. 1996). The interaction of the ET protein that acts as its substrate takes place in the area where the FAD is located as well as in a large surface that belongs to the two domains of the protein (Martínez-Júlvez et al. 1999).

Other enzymes show similar structures to FNR and have been described as belonging to the same structural family. Among them are the bacterial ferredoxin reductases such as flavodoxin reductase from *E. coli* (Ingelman et al. 1997) and also ferredoxin (flavodoxin) reductase (FPR) from *A. vinelandii* (Prasad et al. 1998). There is a group of enzymes that have a number of similar features to FNR but that do not have FAD as a cofactor but they use flavins as substrates.

They are called flavin reductases and their structures are similar to the structures of the ferredoxin reductase family of flavoproteins, despite their very low sequence similarities (Ingelman et al. 1999). Some enzymes combine, together with the FNR-like module, other domains that provides to the enzyme with the corresponding catalytic ability. In phthalate deoxygenase reductase there is a third domain that resembles to plant ferredoxins and contains a [2Fe–2S] cluster (Correll et al. 1992). This is a variant of the previous structure where the interaction between FNR and Fd takes place through the formation of a transient complex. A catalytically competent fragment of maize nitrate reductase has also been resolved and shows the same domains described for FNR. In this case the NADPH and FAD-binding domains are followed linearly by one containing a cytochrome *b* and another one with a molybdopterin cofactor that is where the reduction of nitrate takes place (Lu et al. 1994; Campbell 1999). In *E. coli* sulphite reductase it has been shown that the carboxy-terminal part of the enzyme is composed of an antiparallel β -barrel which binds the FAD, while there is a pyridine dinucleotide binding fold resembling what is typical of the FNR family (Gruez et al. 2000). The three-dimensional structure of NADH cytochrome *b*₅ reductase from pig liver has also shown to present great similarity to that of FNR in spite of the relatively low sequence homology (about 15%) between the two enzymes (Nishida et al. 1995).

Some structural similarities to FNR can also be found in the large family of cytochrome P450 enzymes that are involved in the oxidative metabolism of both endogenous and exogenous compounds including therapeutic drugs and other environmental toxicants and carcinogens. All cytochrome P450 enzymes receive electrons from NAD(P)H through a FAD-containing reductase that in its simplest form use a ferredoxin-type ET protein to connect with the cytochrome. In bacteria this protein is called putida reductase (Poulos et al. 1986) while the one involved in the adrenal synthesis of steroids is called adrenodoxin reductase (Bernhardt 2000). Microsomal P450 enzymes use a reductase that contains a FAD cofactor but it is also connected, through the same polypeptide chain, to a FMN domain that replaces the iron-sulphur protein that participate in bacterial and adrenal P450 enzymes. It is remarkable that both reductases, although performing similar function, belong to a different family. Thus, while the microsomal NADPH-cytochrome P450 reductase (CPR) presents

the same general fold that FNR (Wang et al. 1997), adrenodoxin reductase (Ziegler et al. 1999; Ziegler and Schulz 2000) and the bacterial NADH-dependent ferredoxin reductase component in biphenyl dioxygenase reductase (Senda et al. 2000) show a chain topology that is similar to that of the glutathione reductase family. Moreover, the FMN-binding domain of microsomal cytochrome P450 reductase is also similar to flavodoxin being interesting in this respect because of its resemblance to the algal and cyanobacterial FNR system in which the reductase is coupled transiently to flavodoxin. Finally, the recently described apoptosis-inducing factor (AIF), although physiologically involved in a cell-regulatory process and presenting NADH oxidase activity that leads to the formation of the superoxide ion, shows a structure that differs from that of FNR and must be included in the glutathione reductase family (Mate et al. 2002).

Structural features of *Anabaena* ferredoxin and flavodoxin

Anabaena ferredoxin is an acidic protein that contains a [2Fe–2S] centre inserted into an 11 kDa polypeptide chain. This plant-type ferredoxin participates in the transfer of electrons from Photosystem I (PS I) to FNR. A number of other enzymes, namely nitrate and sulphite reductases, glutamate synthase and thio-reodoxin reductase, receive electrons from ferredoxin to perform their function in cyanobacteria. Its reduction potential has been reported to be in the range of -406 to -440 mV (Hurley et al. 1997) that corresponds to one of the most negative values for a biological molecule. The three-dimensional structure of *Anabaena* ferredoxin has been resolved at high resolution (Rypniewsky et al. 1991; Morales et al. 1999). Most of the residues are inserted into four β -strands that are surrounded by three short α -helices strands. The iron–sulphur centre is located at the edge of the molecule being accessible to electrons exchange.

Anabaena flavodoxin consists of a 168-amino acidic protein that has a non-covalently bound FMN group as the only redox centre. This flavin cofactor is also exposed to the solvent and is susceptible to undergo two one-electron redox reactions giving yield to the formation of the semiquinone species that is not stable when the flavin cofactor is free in solution. The redox potential values for these two reactions are

$E'_{\text{o}_{\text{ox/sq}}} = -195$ mV and $E'_{\text{o}_{\text{sq/rd}}} = -390$ mV (Pueyo et al. 1991) suggesting that it functions between the semiquinone and the reduced form when it is replacing ferredoxin. The three-dimensional structure of *Anabaena* flavodoxin has been reported and it shows to be a α/β protein arranged in an open β -sheet with five parallel β -strands that are surrounded by five α -helices that are in contact with the solvent (Rao et al. 1992).

The molecular basis for the interaction between FNR and ferredoxin (or flavodoxin) could be approached in two different, but complementary, ways: (a) by analysing the three-dimensional structure of the complex formed between the two proteins which requires that crystals with the two proteins forming the physiological complex are available; or (b) by introducing mutations in those residues in the surface of the reductase and of the ET protein and studying the changes that the mutation produces in the kinetic of the electron exchange reaction. We have carried out such studies in both areas during the last 10 years and the main contributions in this particular point are presented in this review.

Interaction of FNR with its protein partners: ferredoxin and flavodoxin

More than 30 years ago Foust et al. (1969) described the formation of complexes between spinach Fd and spinach FNR and suggested the importance that electrostatic interactions should have in the assembling and functionality of these complexes. Chemical modification and kinetic studies confirmed this idea and indicated a strong influence of complementary electrostatic charges on complex formation, stabilisation and subsequent ET, with positive charges on FNR interacting with negative charges on Fd (Zanetti et al. 1979, 1984; Batie and Kamin 1984a, b; Bhattacharyya et al. 1986; Walker et al. 1991; Medina et al. 1992a, b; Jelesarov et al. 1993). Moreover, 10 years ago evidence was also presented, in both the *Anabaena* and the spinach systems, that hydrophobic effects also contributed to the FNR:Fd complex stability (Hurley et al. 1993b; Jelesarov and Bosshard 1994). In recent years many aspects of the interaction of FNR with its protein partners, Fd and Fld, have been extensively characterised using a combination of site-directed mutagenesis, steady-state and transient kinetic measurements, as well as structural determinations by X-ray crystallography.

Critical residues on FNR for the interaction with Fd

Once the three-dimensional structures of the spinach and *Anabaena* FNR species were reported (Karplus et al. 1991; Serre et al. 1996), extensive characterisation of these enzymes on their interaction with Fd was carried out. Non-conservative site-directed mutagenesis of several charged residues on the FNR surface at the concave site of the protein, where its FAD prosthetic group is located and Fd was proposed to bind (Karplus et al. 1991; Serre et al. 1996), clearly illustrated the critical nature of some of these side-chains in the interaction and ET with Fd. Thus, among the positively charged residues analysed in FNR (*Anabaena* numbering); R16, K72, K75 (K88 in spinach), R100 (K116), E139, R264, K290 and K294, those situated in the so-called FAD-binding domain (residues 1–138), which are in close proximity to the FAD, have been shown to play a much more important role than the ones situated in the NADP⁺-binding domain (Table 1) (Aliverti et al. 1994; Martínez-Júlvez et al. 1998a, b, 1999). Thus, positions R16, K72

and, specially, K75 (K88 in spinach) have been clearly shown to be required as positive charge side-chains for efficient interaction with Fd (Aliverti et al. 1994; Martínez-Júlvez et al. 1998a, 1999), while mutations at R100, K138, R264, K290 and K294 hardly cause impairment in the interaction and ET processes between FNR and Fd (Table 1). Analysis of the data also leads to the conclusion that in all these cases altered complex stability is the major determinant of the observed decreased reactivity. Moreover, the FNR structure suggested that not only positive charges, but also some negative charges at the Fd interaction surface might play an important role. Thus, site-directed mutagenesis studies also confirmed that although the carboxylate group of E301 in *Anabaena* FNR (E312 in spinach FNR) is not involved in complex formation with Fd (Table 1), it plays a critical role in the catalytic processes between the isoalloxazine moiety of FAD and the FNR substrates by stabilising the flavin semiquinone intermediate and setting the flavin redox potential. Moreover, this residue seems also implicated in transferring protons from the external medium to the FNR isoalloxazine N5 atom through S80 (Bruns and Karplus 1995; Serre et al. 1996; Aliverti et al. 1998; Medina et al. 1998; Faro et al. 2002a). The structure of the E301A FNR mutant also showed interesting conformational changes in the side-chain of another glutamate residue, E139 (Mayoral et al. 2000). Characterisation of several *Anabaena* E139 FNR mutants suggested that non-conservative replacements produced the formation of less-productive complexes with Fd, and indicate that the nature and the conformation of the residue at position 139 modulates the precise enzyme interaction with the protein carrier (Figures 1D and E) (Hurley et al. 2000; Faro et al. 2002b). Therefore, contribution of both attractive and repulsive interactions is required in achieving the optimal orientation for efficient ET between FNR and Fd.

The contribution of hydrophobic interactions to the stabilisation of the FNR:Fd complex has been analysed in this system, especially because it has been described that they contribute to the stabilisation of other protein–protein complexes (DeLano et al. 2000; Ejdebäck et al. 2000). Thus, the role of several hydrophobic residues, situated on the protein surface in the vicinity of the FAD isoalloxazine ring and in the ‘putative’ Fd-binding site, has been analysed (Martínez-Júlvez et al. 2001). These amino acids, L76, L78 and V136 (in *Anabaena* FNR), are fully conserved in all photosynthetic FNRs from different

Table 1. Effect of replacement of selected FNR charged side-chains on the interaction and kinetic parameters of the FNR:Fd association

| FNR form | $K_d[\text{Fd}_{\text{ox}}:\text{FNR}_{\text{ox}}]^a$ (μM) | k_{et}^b (s^{-1}) | $K_d[\text{Fd}_{\text{rd}}:\text{FNR}_{\text{ox}}]^b$ (μM) |
|------------------------|--|---------------------------------------|--|
| WT ^c | 4 | 3600 | 2.2 |
| Arg16Glu ^d | 120 | n.d. ^e | n.d. ^e |
| Lys72Glu ^d | 50 | n.d. ^e | n.d. ^e |
| Lys75Glu ^d | n.d. ^f | >165 | n.d. ^f |
| Arg100Ala ^g | 10 | 6200 | 9.6 |
| Lys138Glu ^d | 4.1 | 3700 | 1.8 |
| Glu139Lys ^h | 7.3 | 2900 | 12.8 |
| Arg264Glu ^d | 3.3 | 3800 | 1.3 |
| Lys290Glu ^d | 4.0 | 3500 | 1.9 |
| Lys294Glu ^d | 7.2 | 4800 | 2.9 |
| Glu301Ala ^c | 4.6 | 83 | 2.6 |

^a Data obtained by differential spectroscopy in 50 mM Tris/HCl, pH 8.0.

^b Data obtained by laser flash photolysis. In addition to proteins, solution also contained 0.1 mM dRf and 1 mM EDTA in 4 mM potassium phosphate, pH 7.0. Ionic strength was 12 mM.

^c Data from Medina et al. (1998).

^d Data from Gómez-Moreno et al. (1998).

^e Cannot be accurately determined.

^f Complex formation was not detected.

^g Data from Martínez-Júlvez et al. (1998b). These data were obtained at $\mu = 100$ mM.

^h Data from Hurley et al. (2000). These data were obtained at $\mu = 100$ mM.

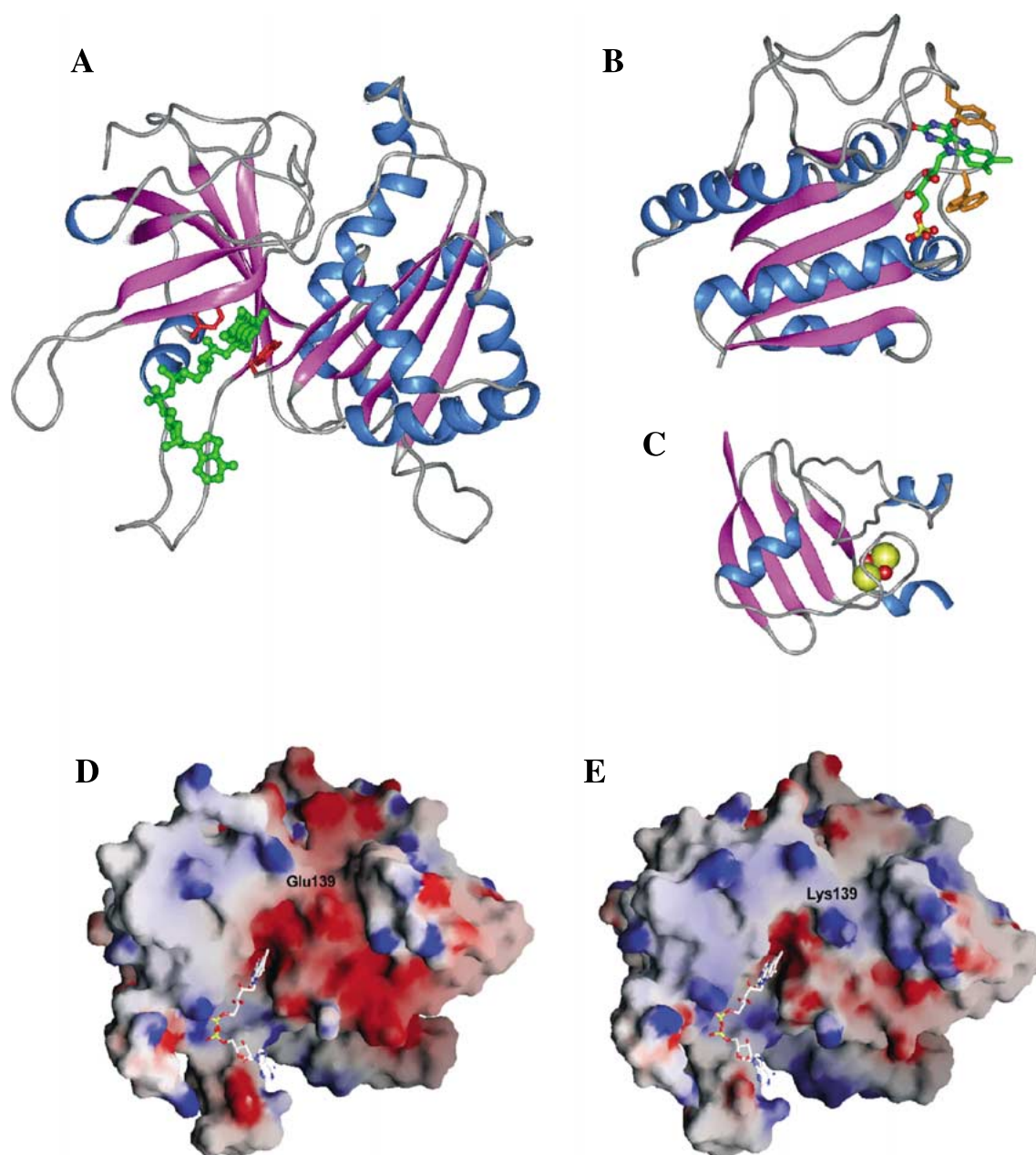


Figure 1. Three-dimensional structures of *Anabaena* FNR, Fd and Fld. Ribbon representation of (A) FNR, (B) Fld and (C) Fd. The FAD (for FNR) and FMN (for Fld) cofactors are shown in sticks representation, as well as the aromatic residues sandwiching their isoalloxazine rings. The [2Fe–2S] cluster of Fd is shown in red and yellow balls. The figures were generated using PREPIPs. Molecular surface showing the electrostatic potentials (D) of WT FNR and (E) the charges introduced in the electrostatic potential around the FAD cofactor by replacement of E139 by a Lys. Positive potential is shown in blue and negative potential in red. The FAD cofactor is represented as sticks. The figure was generated with GRASP.

species. Different ET behaviours with Fd are observed for some of the mutations made at L76, L78 and V136. Thus, the ET interaction with Fd is almost completely lost upon the introduction of negatively charged

side-chains. However, subtle changes in the hydrophobic patch can influence the rates of ET to and from Fd by altering the binding constants and the midpoint redox potentials of the flavin group (Table 2).

Table 2. Effect of replacement of selected FNR hydrophobic side-chains on the interaction and kinetic parameters of the FNR:Fd association^a

| FNR form | E_m (mV) | K_d [Fd _{ox} :FNR _{ox}] ^b (μ M) | k_{et}^c (s ⁻¹) | K_d [Fd _{rd} :FNR _{ox}] ^c (μ M) |
|----------|------------|---|-------------------------------|---|
| WT | -325 | 4 | 6200 | 9.3 |
| Leu76Asp | -330 | n.d. ^d | n.d. ^e | n.d. ^e |
| Leu76Val | | 3.3 | 3700 | 9.0 |
| Leu76Phe | -333 | 7.6 | n.d. ^e | n.d. ^e |
| Leu76Ser | -305 | 11 | 8400 | 12.2 |
| Leu78Asp | -302 | n.d. ^d | 2000 | 18.8 |
| Leu78Val | | 2.9 | 6700 | 22.0 |
| Leu78Phe | -307 | 27.4 | n.d. ^e | n.d. ^e |
| Leu78Ser | -286 | 6.3 | 8100 | 14.1 |

^a Data from Martínez-Júlvez et al. (2001).

^b Data obtained by differential spectroscopy in 50 mM Tris/HCl, pH 8.0.

^c Data obtained by laser flash photolysis in 4 mM potassium phosphate, pH 7.0 containing 0.1 mM dRf and 1 mM EDTA. Ionic strength was adjusted to 100 mM.

^d Complex formation was not detected.

^e Cannot be determined.

Therefore, it has been shown that, specially, L76 and L78 are critical in the binding and orientation of Fd for efficient ET (Martínez-Júlvez et al. 2001).

Critical residues on Fd for the interaction with FNR

Similar results to those above shown for FNR have been reported for charge-reversal mutations on the Fd surface. Thus, charge-reversal of side-chains such as D62, D67, D68, D69 or E95 (*Anabaena* Fd numbering) have only a moderate effect on complex stability and ET with the reductase (Hurley et al. 1993a). In contrast, a charge-reversal mutation of E94 has large effects on the ET reactivity (Hurley et al. 1993a, 1994, 1997). The crucial role of E94 has been discussed in terms of the Fd X-ray structure that shows this residue forming a hydrogen bond with the hydroxyl group of S47, which has also been shown to be a critical residue. Such studies suggest an important role of these two residues in the stabilisation of the molecular surface of the Fd. It is noteworthy that the adjacent residue, E95, cannot carry out the function played by E94. Moreover, in the FNR/Fd system it has also been shown that an aromatic amino acid residue on the surface at position 65 of *Anabaena* Fd (F65 in the native enzyme) is essential for the efficient ET reaction between Fd and FNR (Hurley et al. 1993a, b). In this case, again the introduction of an aromatic residue in the preceding position could not substitute for the function of an aromatic residue at position of F65.

These facts indicate the importance of the geometry, the precise complementarity and the specific nature at the interface between FNR and Fd.

The three-dimensional structure of the Fd:FNR interaction

The site-directed mutagenesis results above described can be compared with the three-dimensional structures recently reported for the FNR:Fd complexes from *Anabaena* and maize (Morales et al. 2000; Kurisu et al. 2001). When the FNR molecules of the *Anabaena* and maize complexes are superimposed the Fd partners appear rotated by an angle of 95°. Nevertheless, in both complex structures the [2S-2Fe] cluster of Fd and the FAD of FNR are in close proximity

Table 3. Intermolecular salt bridges that are able to be formed at the interface of the FNR:Fd complex according to the three-dimensional structures reported for the *Anabaena* and maize FNR:Fd complexes

| FNR:Fd <i>Anabaena</i> complex ^a | | FNR:Fd maize complex ^b | |
|---|-------------|-----------------------------------|-------------|
| Fd residue | FNR residue | Fd residue | FNR residue |
| Glu94..... |Lys75 | Asp65..... |Lys91 |
| Glu95..... |Lys72 | Asp66..... |Lys88 |
| Asp59..... |Lys293 | Glu29..... |Lys304 |
| Asp23..... |Lys290 | Arg40..... |Glu154 |
| Asp67..... |Arg16 | Asp61..... |Lys33 |

^a Data from Morales et al. (2000).

^b Data from Kurisu et al. (2001).

(at 6 Å in the maize and 7.4 Å in the *Anabaena*) and electrostatic intermolecular interactions, occurring through salt bridges, are involved in the outer complex interface (Table 3). Moreover, in both cases the interface around the prosthetic groups is hydrophobic (Figure 2B). Thus, in the *Anabaena* FNR:Fd complex the molecular interface consists of a core of hydrophobic side-chains including mainly L76, L78 and V136 on the FNR surface and F65 on the Fd one. In the case of the maize complex, such region is formed by four residues on FNR, V92, L94, V151 and V133, being the first three equivalent in sequence and position to L76, L78 and V136 of *Anabaena* FNR. However, with regard to the Fd, although the equivalent residue to *Anabaena* Fd F65 in the maize Fd, Y63, is situated at the hydrophobic interface between FNR and Fd, in the three-dimensional structure of the maize complex this residue side chain is neither in close contact with the [2Fe–2S] cluster, nor is between the two prosthetic groups as shown in the *Anabaena* complex (Morales et al. 2000; Kurisu et al. 2001). In the outer sphere of this hydrophobic core a well-defined salt bridge is observed in both complexes, between FNR K75 and Fd E94 in the *Anabaena* complex and between the corresponding FNR K91 and Fd D65 in the maize one. Additionally, charged groups from both proteins interact either directly or through water molecules (Table 3). In the case of maize these salt bridges are clearly formed and the residues involved in those patches are K33, K88, K91, E154 and K304 of maize FNR. The structure of the *Anabaena* interaction also shows that the equivalent residues on FNR, R16, K72, K75, E139 and K293, are able to form salt bridges with Fd in a similar manner (Table 3). It is worth to note that although FNR contributes to the interaction mainly with positively charged residues, the three-dimensional structure of the maize complex shows that FNR E154, a negative residue, has a strong interaction with R40, a basic residue from Fd. As shown above, the equivalent residue in *Anabaena* FNR has been shown to be involved in optimal complex orientation (Faro et al. 2002b). Moreover, other hydrophobic forces, either involving long carbonyl side-chains (like R264 in *Anabaena* FNR), or resulting from the loss of water molecules at the complex interface, are also shown to contribute to the complex stability of the crystal structures (Figure 2). Therefore, despite small differences between some side chains of maize and *Anabaena* FNR, both crystallographic complexes indicate that they use the same surface region for the interaction with Fd. Moreover, the residues

involved in such interaction are equivalent in both systems, and also, are those predicted by site-directed mutagenesis studies.

Critical residues on FNR for the interaction with Fld

Since in some photosynthetic systems, like that of certain algae or cyanobacteria, Fld can efficiently replace Fd in the overall ET process, a careful analysis of the FNR:Fld interaction has also been carried out in the case of the *Anabaena* proteins. The fact that both proteins have different size, structure and redox cofactor and, still, they interact at the same site, suggest that comparison of the interface area in both proteins could provide important information about the determinants for molecular recognition. Analysis of several charge-reversal mutants on the FNR surface in the *Anabaena* system indicated that the positively charged residues situated within the FNR FAD-binding domain, which have above been shown to be critical for achieving maximal ET rates with Fd, R16, K72 and, specially K75, are also determinant for an efficient interaction with Fld (Martínez-Júlvez et al. 1998a, 1999). However, with regard to the positively charged residues studied at the NADP⁺-binding domain, it has been shown that K138 and R264 side chains are more important in establishing interaction with Fld than with Fd (Table 4) (Martínez-Júlvez et al. 1998b, 1999). The role of the FNR negatively charged side chains E301

Figure 2 Three-dimensional structure of the *Anabaena* FNR:Fd complex. (A) GRASP transparent molecular surface representation superimposed onto a MOLSCRIPT drawing of the *Anabaena* FNR:Fd complex showing the secondary structure elements. The FNR N-terminal FAD binding domain is shown in yellow, the FNR C-terminal NADP⁺ binding domain in blue and Fd in red. The FAD and the [2Fe–2S] redox centres are shown as black lines. (B) A detailed view showing the electron density and the side-chain interactions in the redox centres environment. Electron density is shown in red for Fd and green for FNR.

Figure 3 FNR:NADP⁺ interaction. (A) FAD and NADP⁺ binding sites in FNR:NADP⁺ C-II. The protein surface is shown in yellow and FAD (orange) and NADP⁺ (CPK colour) are shown in sticks. A narrowing of the cavity in which the 2'-P-AMP and the pyrophosphate are accommodated is observed upon complex formation. (B) Relative positions of isoalloxazine (CPK colour) and nicotinamide rings in the C-I (red), C-II (yellow) and C-III (green) FNR:NADP⁺ complexes. (C) Putative model for a transient Fd:FNR:NADP⁺ ternary complex in *Anabaena*. This model was obtained by superposition of the FNR coordinates in the Fd:FNR complex with those in the FNR:NADP⁺ C-II complex. Molecular surface of Fd is shown in blue and that of FNR in grey. The FAD and NADP⁺ cofactors are in CPK colour in sticks.

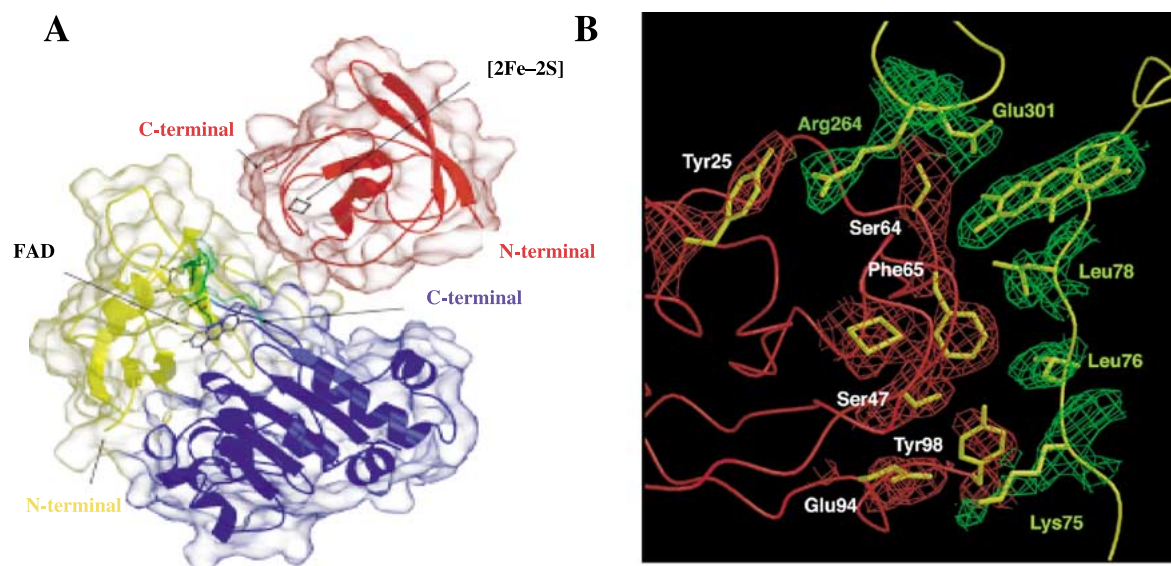


Figure 2

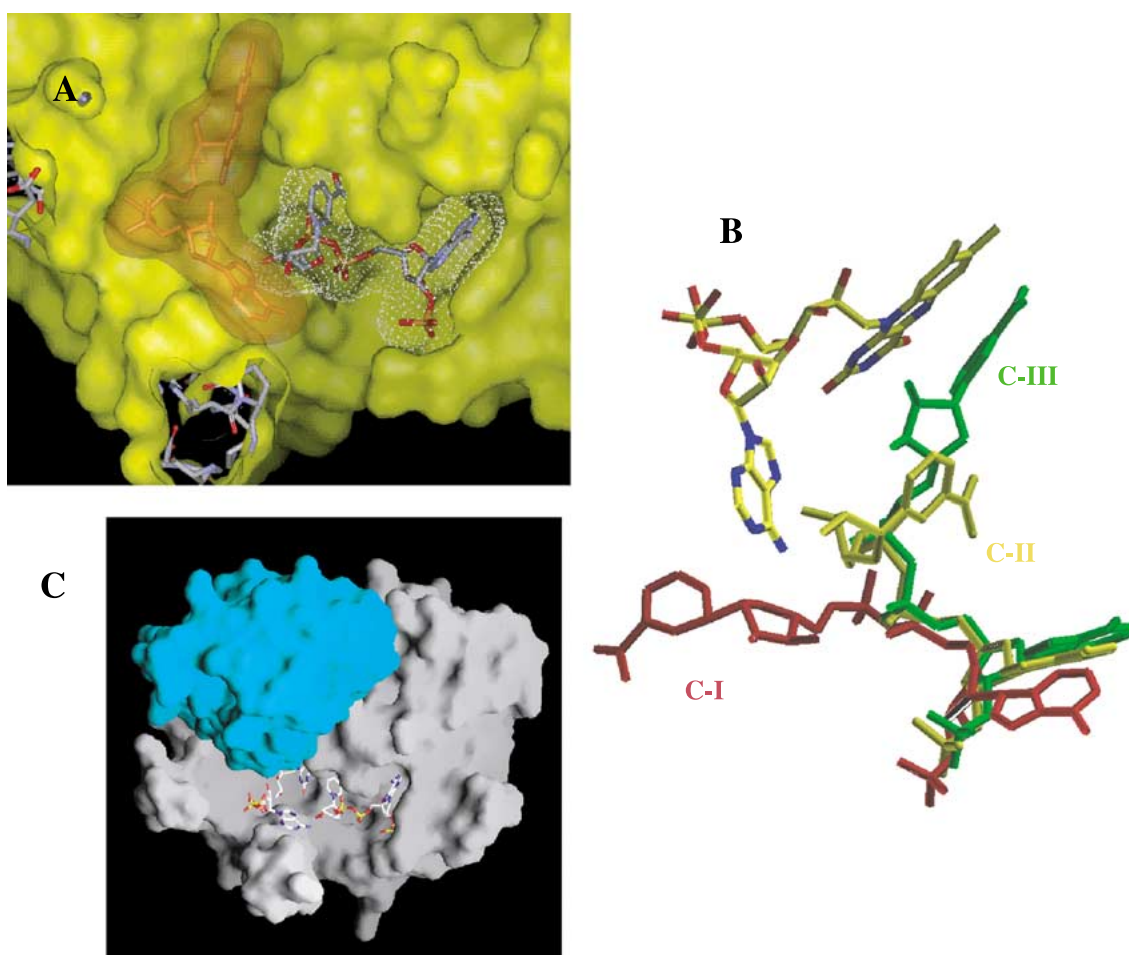


Figure 3

and E139 in the interaction and ET processes with Fld has also been analysed. As happened in the processes with Fd, E301 in *Anabaena* FNR is not involved in complex formation with Fld, but rather plays a role in the catalytic processes (Table 4) (Medina et al. 1998; Mayoral et al. 2000; Faro et al. 2002a). With regard to E139, its side chain has been shown to play an active role in Fld interaction, since either removal of the negative charge or charge-reversal mutations reduce

Table 4 Effect of replacement of selected FNR charged side-chains on the interaction and kinetic parameters of the FNR:Fld association^a

| FNR form | $K_d[\text{Fld}_{\text{ox}}:\text{FNR}_{\text{ox}}]$ (μM) | $k_{\text{ap}} \text{Fld}_{\text{rd}} + \text{FNR}_{\text{ox}}$ (s^{-1}) | K_m^{Fld} (μM) |
|------------------------|---|---|---|
| WT ^b | 3 | >600 250 | 33 |
| Arg16Glu ^c | n.d. ^d | 1.6 1.5 | >500 |
| Lys72Glu ^c | n.d. ^d | 13 3.4 | 283 |
| Lys75Glu ^c | n.d. ^d | 0.52 0.08 | n.d. ^e |
| Arg100Ala ^f | 3.4 | >800 | 78 |
| Lys138Glu ^c | n.d. ^d | 31 13 | 310 |
| Glu139Lys ^g | – | >800 | 10 |
| Arg264Glu ^c | 9.4 | 16.6 3.1 | 544 |
| Lys290Glu ^c | 7.1 | 83 11.5 | 183 |
| Lys294Glu ^c | 20.3 | 165 43 | 125 |
| Glu301Ala ^b | 4 | 35 10 | 11.9 |

^a Reactions in Tris/HCl 50 mM, pH 8.0 ($\mu = 28 \text{ mM}$). K_d were determined by differential spectroscopy, k_{ap} by stopped-flow and K_m^{Fld} by measurement of the FNR steady-state cytochrome *c* reductase activity.

^b Data from Medina et al. (1998).

^c Data from Gómez-Moreno et al. (1998).

^d Complex formation was not detected.

^e Activity too low to be determined.

^f Data from Martínez-Júlvez et al. (1998b). The k_{ap} value for ET occurred within the instrument's dead time.

^g Data from Faro et al. (2002b). The K_d value for this interaction has not been reported and the k_{ap} value for ET occurred within the instrument's dead time.

the possible orientation modes between FNR and Fld to more productive and stronger ones (Faro et al. 2002b). Hence, although E139 of *Anabaena* FNR modulates the precise enzyme interaction with the protein carriers, Fd and Fld, this residue behaves in a different way with both ET proteins, since removal of the negative charge at position 139 of *Anabaena* FNR produces a deleterious effect in its ET reactions with Fd whereas it appears to enhance the ET processes with Fld (Tables 1 and 4). With regard to the hydrophobic interactions different ET behaviours with Fld are also observed for some of the mutations made at the hydrophobic patch formed by L76, L78 and V136 of *Anabaena* FNR (Table 5) (Nogués et al. 2003). Thus, the ET interaction with Fld is almost completely lost upon introduction of negatively charged side-chains at these positions, while, as happened in the reaction with Fd, more conservative changes in the hydrophobic patch modulate the ET rates by

Table 5 Effect of replacement of selected FNR hydrophobic side-chains on the interaction and kinetic parameters of the FNR:Fld association^a

| FNR form | $K_d[\text{Fld}_{\text{ox}}:\text{FNR}_{\text{ox}}]$ (μM) | $k_{\text{ap}} \text{Fld}_{\text{rd}} + \text{FNR}_{\text{ox}}$ (s^{-1}) | K_m^{Fld} (μM) |
|----------|---|--|---|
| WT | 3 | >600 250 | 33 |
| Leu76Asp | n.d. ^b | 6.1 1.6 | n.d. ^d |
| Leu76Val | 102 | 170 19 | n.d. ^c |
| Leu76Phe | 7.8 | 46 7 | n.d. ^c |
| Leu76Ser | 6.7 | 42 0.5 | n.d. ^d |
| Leu78Asp | n.d. ^b | 0.5 0.14 | n.d. ^d |
| Leu78Val | 10.7 | >400 140 | 52 |
| Leu78Phe | 17 | 28 3.6 | n.d. ^c |
| Leu78Ser | 31.7 | 24 | n.d. ^d |

^a Data from Martínez-Júlvez et al. (2001). Measurements performed in 50 mM Tris/HCl pH 8.0.

^b Complex formation was not detected.

^c Cannot be determined.

^d No reaction was detected.

altering the binding constants and the midpoint redox potentials of the flavin group. Therefore, it is concluded that the above non-polar residues of FNR also participate in the establishment of interactions with Fld, as was also the case with Fd. However, as above already shown, most of the mutations produced slight different behaviours with both ET proteins. Therefore, it can be proposed that Fld occupies the same region than Fd for the interaction with the reductase. However, each individual residue on the FNR surface does not participate to the same extent in the processes with Fd and Fld.

Critical residues on Fld for the interaction with FNR

Although, as shown above, some residues on *Anabaena* FNR, either polar or non-polar, have been proved to be critical for an optimal interaction between the reductase and Fld (Tables 4 and 5) (Martínez-Júlvez et al. 1998a, 1999; Nogués et al. 2003), thus far there is no candidate residue on the Fld surface that appears to interact with them. Early chemical modification studies suggested the involvement of some negatively charged residues on the *Anabaena* Fld surface (Medina et al. 1992c). However, later analysis of site-directed mutants at positions of residues E61, E67, D100, D126, D144 and E145 (Navarro et al. 1995; Jenkins et al. 1997) indicated that none of them appears to be critical for the complex formation and ET processes between Fld and its substrates. In an attempt to look for the FNR hydrophobic counterpart region on the Fld surface, the effect produced by the replacement of the only two non-polar residues on the Fld surface in the FMN surroundings, I59 and I92, by a Lys has also been analysed. The results obtained suggest that these two hydrophobic residues are not critical in the interaction and ET processes with FNR (Nogués et al. 2003). However, significant effects are observed in these I92- and I59-Fld mutants with respect to their ability to accept electrons from the membrane-anchored PS I complex, specially for the I92 position of Fld, both in the processes of association and ET (Nogués et al. 2003). The analysis of the influence of the amino acid residues sandwiching the FMN ring in *Anabaena* Fld in complex formation and ET with PS I and FNR has also been examined in several mutants of the key residues W57 and Y94 (Casaus et al. 2002). These mutants' ability to form complexes with either FNR or PS I is similar to that of wild-type Fld. Thus, although some of the mutants

exhibit altered kinetic properties in their ET processes, they can be explained in terms of altered flavin accessibility and/or thermodynamic parameters (Lostao et al. 1997; Casaus et al. 2002). Therefore, the overall data indicated that W57 and Y94 do not play any active role in Fld interaction and ET with FNR. It is proposed that these residues are rather setting an appropriate structural and electronic environment that modulates *in vivo* ET from PS I to FNR while provide a tight FMN binding (Casaus et al. 2002). In conclusion, thus far, site-directed mutagenesis studies have not revealed any key residue on the Fld surface for the interaction with FNR and, therefore, no information has been obtained by this technique about the orientation or the way of Fld binding to FNR prior to ET.

The three-dimensional model of the FNR:Fld interaction

Despite much effort in obtaining a refracting crystal, thus far the three-dimensional structure of the FNR:Fld complex remains unsolved. The FNR:Fld complex crystallisation appears to be complicated due not only to the size of the proteins, but also to the nature of the interaction. This might indicate that the interactions formed between FNR and Fld are short-lived and of low stability in order to favour a fast ET process. Biochemical studies clearly suggest that the FNR uses the same surface for interaction with Fd and Fld. In many cases, it has also been observed that the residues involved in the interaction with Fd are the same that those responsible for the binding of Fld (Martínez-Júlvez et al. 1999, 2001; Nogués et al. 2003). On the other hand, the analysis of Fd and Fld surface electrostatic potentials shows an overlapping that suggests a similar way of interaction with the reductase for both of them (Ullmann et al. 2000).

As mentioned above, the three-dimensional structure reported for microsomal CPR might be used as a model for the FNR:Fld interaction (Wang et al. 1997). This enzyme contains both FAD and FMN and is composed of four structural domains: the FMN-binding domain, the connecting domain, and the FAD- and NADPH-binding domains. The FMN-binding domain is similar to the structure of Fld, whereas the FAD- and NADPH-binding domains are similar to those of FNR. The connecting domain, situated between the Fld- and FNR-like domains, is responsible for the relative orientation of the other domains, ensuring the proper orientation of the two flavins for efficient ET. Noticeably,

in this arrangement the two flavin isoalloxazine rings are juxtaposed, being the closest distance between them about 4 Å. Taking into account that in FNR and Fld the methyl groups of the flavin ring are exposed to the solvent, the simple superposition of FNR and Fld onto the CPR structure would leave their flavin redox centres to a much closer distance than that observed in the FNR:Fd structures (about 7.6 Å) (Morales et al. 2000; Kurisu et al. 2001). Therefore, considering that the proximity of the redox centres in a non-polar environment is crucial for efficient ET between proteins, the FNR:Fld complex would exchange electrons more easily than the FNR:Fd one. Consequently, the orientation of the ET flavoprotein on the FNR surface does not affect the relative distance between the methyl groups of the FAD and FMN as much as occurs in the complex with Fd. Such observations would fully agree with the fact that, whereas multiple chemical modification produced Fld forms less suitable for ET than the unlabelled one (Medina et al. 1992c), site-directed mutagenesis on individual residues has not revealed any critical residue on the Fld surface for the interaction with FNR. This would also explain the difficulties in obtaining a crystal structure for the FNR:Fld interaction. Thus, while FNR and Fd should establish very specific interactions, either electrostatic or hydrophobic, between their respective surface side-chains, the FNR:Fld interaction might present less geometric requirements for an efficient ET. Such difference in specificity has also been recently pointed out in the case of the FNR from *E. coli*, enzymes that accept electrons from NADPH in order to reduce either Fd or Fld1 or Fld2 to provide low-potential electrons (Wan and Jarrett 2002). In this FNR it has been clearly shown that efficiency of ET from FNR to any of the Flds is much lower than that to Fd. Noticeably, it is also shown how, despite important differences in residues surrounding the flavin cofactor in Fld1 and Fld2, both of them behave indistinguishable with regard to FNR, suggesting less specificity in the FNR:Fld interaction. Moreover, although different crystal structures have also been reported for the flavo-protein monomer of the sulphite reductase (SiR-FP), equivalent to CPR, in all of them only the FNR-like module is present in the density maps despite a functional FMN-binding module is present in the protein (Gruez et al. 2000). These observations clearly indicate a disordered Fld-like module, suggesting that in SiR-FP affinity between FNR-like and Fld-like modules must be lower than in the CPR and, that flexibility of the Fld-like domain could be related to a reorganisation of the domains in the course of ET. Therefore,

having into account all these observations and results so far reported for the photosynthetic FNR/Fd and FNR:Fld system, a less specific interaction between FNR and Fld, with regard to the FNR/Fd interaction, must be expected. However, further work must be done in the comparative study of these two systems in order to make this point clear.

Interaction of FNR with its coenzyme: NADP⁺/H. Specificity for the coenzyme

Critical residues on FNR for the interaction with NADP⁺

Early chemical modification studies implicated K116 and K244 in the spinach FNR (corresponding to R100 and R233 in the *Anabaena* enzyme) (Chan et al. 1985; Cidaria et al. 1985; Aliverti et al. 1991a), and R224, K227 and R233 (Medina et al. 1992a, b) in the *Anabaena* enzyme, in the interaction with NADP⁺. Shortly after, site-directed mutagenesis studies probed the critical role of the equivalent side-chains, K116 and R100 in spinach and *Anabaena* FNRs, respectively, in the binding and correct placing of NADP⁺ for catalysis (Aliverti et al. 1991b; Martínez-Júlvez et al. 1998b).

Recent site-directed mutagenesis studies on *Anabaena* FNR also confirmed the role played by the S223, R224, R233 and Y235 in the stabilisation of the 2'-P-AMP moiety of the coenzyme (Medina et al. 2001). These studies also suggest that the determinants of the high specificity shown by this enzyme for NADP⁺/H versus NAD⁺/H is not only provided by these residues directly interacting with the 2'-P of NADP⁺/H and, that other regions of the protein must undergo specific structural arrangements to determine coenzyme specificity (Medina et al. 2001). Characterisation of an *Anabaena* FNR mutant in which T155, a residue conserved in all NADP⁺/H-dependent members of the family, had been replaced by Gly, a residue present in all NAD⁺/H-dependent members, produced important changes in a region (the 261–265 loop) rich in Pro residues, therefore having a high degree of rigidity, in the case of the NAD⁺/H-dependent enzymes but not in the NADP⁺/H-dependent enzymes. Finally, in the case of the pea enzyme it has been shown that replacement by site-directed mutagenesis of the C-terminal tyrosine, Y308 (Y303 in *Anabaena*), residue that it is distant from the 2'-P group of NADP⁺/H, produced FNR forms in which the preference for NADP⁺/H over NAD⁺/H was considerably

decreased (Piubelli et al. 2000). These data suggest that the C-terminal tyrosine enhances the specificity for NADP⁺/H by destabilising the interaction of the nicotinamide ring of both NADP⁺ and NAD⁺. Therefore, they again support the hypothesis that specific recognition of the 2'-P group of NADP⁺/H by FNR is required but it is not sufficient to ensure a high degree of discrimination for the coenzyme.

The three-dimensional structure of the FNR:NADP⁺ complex

Complexes of FNR forms, from *Anabaena* and pea species, with NADP⁺ have also been studied by X-ray crystallography revealing different orientations for the nicotinamide location of the coenzyme with regard to the isoalloxazine ring (Serre et al. 1996; Deng et al. 1999; Hermoso et al. 2002). In all these complexes the adenine ring of NADP⁺ is hydrogen bonded with Q237 and is stacked with the Y235 side-chain, whereas the 2'-P interacts with S223, R224, R233 and Y235. The adenine 5'-phosphoryl is hydrogen bonded to R100, but different conformers are observed for this residue in the different complexes. Therefore, all these structures provide reliable binding information for the 2'-P-AMP coenzyme moiety that is compatible with the chemical modification and site-directed mutagenesis studies.

The first *Anabaena* FNR:NADP⁺ complex (C-I) reported was obtained by soaking native FNR crystals in an NADP⁺ solution (Figure 3B) (Serre et al. 1996). In this complex, no changes were detected in the protein backbone with regard to the free enzyme, and only slight rotations were observed for the side chain of some residues in order to approximate to the 2'-P group. However, the nicotinamide appeared turned towards the molecular surface, far away from the FAD isoalloxazine ring. Three years later, the three-dimensional structure of a complex between NADP⁺ and a pea FNR mutant, in which the C-terminal Tyr residue had been replaced by Ser, was reported (C-III) (Figure 3B) (Deng et al. 1999). In this complex, the coenzyme shows a characteristic L shape with the pyrophosphate group located at the corner and the two branches of the L accommodating the NMN moiety and the 2'-P-AMP, respectively. The adenine moiety of the NADP⁺ is sandwiched between the hydrophobic L263 side-chain and the aromatic ring of Y235 (*Anabaena* numbering). The pyrophosphate moiety of NADP⁺ is essentially stabilised by the guanidine group of R100, which presents a dif-

ferent conformer than C-I. Finally, removal of the terminal Tyr (Y303 in *Anabaena*) allows the approach of the nicotinamide group to the FAD isoalloxazine ring, which situates its C4 atom at only 3 Å from the N5 atom of the isoalloxazine ring.

Recently, another model for the FNR:NADP⁺ interaction has been reported by cocrystallisation of *Anabaena* WT FNR with NADP⁺ (C-II) (Figures 3A and B) (Hermoso et al. 2002). In this complex the pyrophosphate and 2'-P-AMP portions of NADP⁺ present a similar conformation to the analogous moieties of the C-III and are stabilised by equivalent interactions with the protein. Y303 is stacked between the isoalloxazine and the nicotinamide rings. Therefore, it is remarkable that although the nicotinamide ring of the NADP⁺ is placed near the isoalloxazine ring, it does not replace the C-terminal Tyr as required for efficient ET. The nicotinamide moiety is only stabilised by two polar interactions with T155 and by a van der Waals contact with L263. Finally, in this complex the ribose of the NMN moiety is found in a very tight conformation.

Although three structural different binding modes have been shown for the NADP⁺:FNR interaction, none of them represents a functional model for the *in vivo* hydride transfer process (Figure 3B). Nevertheless, none of these structures excludes the others and they have been interpreted as different steps in the molecular recognition and complex reorganisation to provide the adequate orientation for the hydride transfer (Hermoso et al. 2002). Thus, C-I might represent the initial recognition of NADP⁺ as an FNR coenzyme, through its 2'-P-AMP moiety and the 221–239 region of the protein. C-II will be then produced by a rearrangement that includes a better stabilisation of the 2'-P-AMP moiety by the 221–239 region, a change in the conformation of R100 to H-bond the pyrophosphate bridge, and, a change in the conformation of the 261–268 region to accommodate and to stabilise the adenine and nicotinamide rings. These protein structural rearrangements are associated with a change in the NADP⁺ molecule conformation, which goes from the extended conformation observed in C-I to the more tighten one observed in C-II. At this stage, the ribose and nicotinamide groups are in a tight conformation, which will allow the nicotinamide ring to approach the isoalloxazine after Y303 displacement, as can be observed in C-III.

The key point in this mechanism is: how is the C-terminal Tyr removed from its stacking position with the flavin ring in order to allow the entrance of the nicotinamide ring in this position? The driving

force for such displacement still remains unknown, although some hypothesis can be made. Since the aromatic stacking interactions between activated rings seem to be destabilising (Hunter and Sanders 1990), it has been suggested that the stacking interaction between the Tyr and the isoalloxazine rings may even be hindered upon FAD reduction, and Y303 displacement could be a direct consequence of both the isoalloxazine reduction and the proton transfer process. Thus, the C-terminal Tyr can make way for the nicotinamide ring to become located at the active site, thereby allowing hydride transfer, and then down-modulating the affinity for NADPH, making the release of NADPH feasible due to the stronger affinity of Tyr for the oxidised isoalloxazine ring (Deng et al. 1999; Hermoso et al. 2002). However, this mechanism might only provide for catalysis in the direction of NADP⁺ reduction, while FNR has also been shown to be very active in NADPH oxidation (Carrillo and Ceccarelli 2003). In this case, the modulation of C-terminal Tyr movement upon reduced nicotinamide binding seems less easy to understand. Nevertheless, different pathways are accepted for the forward and backward reactions of FNR, suggesting that different kinetic constraints are present for them. Moreover, it is also proposed that Fd binding might also be involved in the displacement of the C-terminal tyrosine by providing a new hydrophobic environment for the phenol ring. Thus, although thus far it is assumed that the C-terminal Tyr will move from his position during FNR catalytic mechanism, further work must be done in order to understand this mechanism.

The role of this terminal Tyr and the features in the environment of the FAD in the kinetic properties of FNR can be also envisaged by comparison with other enzymes. Thus, it has been shown that this tyrosine residue stacked on the isoalloxazine ring in the NADPH binding site is absent in some enzymes with an overall folding homologous to FNR. An example is FPR from *A. vinelandii*, an enzyme involved in the response to oxidative stress that transfers electrons from NADPH to Fd or Fld, and that lacks this Tyr residue, its position being occupied by an Ala, which carbonyl group interacts with N10 of the flavin (Sridhar et al. 1998). Moreover, FPR presents an extension of the C-terminus by four residues relative to FNR, whereas the equivalent reductase from *E. coli* presents a single additional aromatic residue (Ingelman et al. 1997; Wan and Jarrett 2002). In both of these enzymes the first residue on this extra region is an aromatic residue that stacks on the adenine

ring of FAD forcing a folded over conformation for this nucleotide that differs from the extended L conformation observed in FNR. Both of these enzymes present reduction rates considerably slower than FNR, suggesting that probably their extended regions at the C-terminal produce a slower adaptation of the coenzyme, which has been used in order to regulate the rate of ET process.

On the other hand, superposition of the NADP⁺ binding domain of these FNR: NADP⁺ complexes with the corresponding domain of several NAD⁺/H-dependent members of the FNR family shows that NADP⁺ binding induces conformational changes for the 261–265 loop that make it more similar to that region of the NAD⁺/H-dependent members of the FNR family (Hermoso et al. 2002). These observations clearly suggest different mechanisms for coenzyme recognition and complex reorganisation in FNR, and therefore in NADP⁺/H-dependent members of the FNR family relative to the NAD⁺/H-dependent members. Thus, in the case of FNR, it seems that the free enzyme presents a large cavity to accommodate the 2'-P-AMP moiety of the coenzyme which, upon its binding, is reorganised in order to perfectly match the charge and shape of the adenine portion of the substrate. However, in the case of the NAD⁺/H-dependent members, such a narrow cavity is already preformed in the free enzyme and probably does not need to undergo important structural rearrangements in order to adapt the adenine moiety of NAD⁺/H.

Interaction and electrons transfer in the Fd:FNR:NADP⁺ ternary complex: the overall reaction pathway

Fast kinetic, binding equilibrium and steady-state studies suggested that the FNR-dependent ET from Fd to NADP⁺ must proceed through the formation of a transient ternary complex (Batie and Kamin 1984a, b, 1986). Thus, the first pathway mechanism for the FNR-mediated ET was formulated proposing that Fd bound to a preformed FNR:NADP⁺ complex (Batie and Kamin 1984a, b). Subsequently the binding and kinetic parameters for several of the individual steps proposed in this mechanism have been thoroughly analysed. A recent manuscript by Carrillo and Ceccarelli (2003) provides an updated revision of the current knowledge of each one of the individual steps of the mechanism proposed by Batie and Kamin (1984). This revision makes also an extensive

description of the various key features in the FNR catalytic mechanism that still remain not explained. Therefore, the present section will only give some additional information and the reader is referred to the mentioned manuscript for a more detailed description of each individual step in this mechanism (Carrillo and Ceccarelli 2003).

Although it is not clear how the structural changes observed in the crystal structures so far reported for FNR in complex with its substrates, especially in the active site region, must correlate with the thermodynamic properties of the prosthetic groups upon changing their microenvironments and/or with the induced-fit mechanism deduced from kinetic measurements, the analysis of the structure of a ternary complex is of great interest. Thus, on the basis of the *Anabaena* Fd:FNR crystal structure (Morales et al. 2000) and that of the *Anabaena* NADP⁺:FNR (C-II, Hermoso et al. 2002), and on that of the maize Fd:FNR (Kurusu et al. 2001) and the pea NADP⁺:Y308SFNR (C-III, Deng et al. 1999), putative Fd:FNR:NADP⁺ ternary complexes have been modelled (Hermoso et al. 2002; Carrillo and Ceccarelli 2003). Both models show that the NADP⁺ binding site on FNR is not close to the Fd:FNR interface and, therefore, previous NADP⁺ binding to FNR, although producing some reorganisation in the side-chains interacting with the NADP⁺ coenzyme, should not affect the interaction between the two proteins. Moreover, upon NADP⁺ binding, structural rearrangements in the 261–265 loop of FNR are observed that, in the case of the *Anabaena* complex, involve changes in the conformation and orientation of R264 (Hermoso et al. 2002). Such differences might explain why the affinity of Fd is weaker for the preformed FNR:NADP⁺ complex than for the free enzyme, thus facilitating Fd release once the cycle is completed. Moreover, in the case of the maize complex it has been reported that on Fd:FNR complex formation, the NADPH binding FNR domain is displaced and the side-chain of E312 (E301 in *Anabaena* FNR) moves to hydrogen bonding distance of the hydroxyl group of S96 (S80 in *Anabaena*) (Kurusu et al. 2001). Noticeably, early analysis of the FNR crystal structure already suggested that S96 would be directly involved in hydride transfer between the isoalloxazine moiety of FAD and the nicotinamide ring of NADP⁺/H, whereas E312 might be involved in providing the proton (Karplus and Bruns 1994; Serre et al. 1996). Site-directed mutagenesis has confirmed the crucial role of these residues

for electron and hydride transfer (Aliverti et al. 1995, 1998; Medina et al. 1998; Mayoral et al. 2000).

Concluding remarks

The availability of the three-dimensional structures of the individual proteins, has prompted the scientific community interested in the study of the mechanism of ET to try to understand how two proteins recognise each other and interact to exchange electrons. All data available indicate that the reaction takes place through the formation of a transient complex that, once the reaction has taken place, is disassembled and the reaction starts again. The use of site-directed mutagenesis, together with precise kinetic measurements, allows ascribing a specific function to certain residues of the proteins. So, charged amino acid residues on the surface provide the protein with a dipole moment that helps orienting it and making possible the first encounter between the proteins. But the mutual disposition that the two proteins acquire after this first step is not necessarily the optimal for electron exchange and requires that the two proteins reorient in order to get the redox centres closer together and with the right orientation for the ET reaction. Such reorganisation is proposed to take place with the involvement of hydrophobic residues and the release of a number of water molecules on the surface of the proteins that move away upon complex formation. The releases of the water molecules, together with the hydrophobic interactions that are then formed provide the energy required for complex formation. One peculiarity of some of the proteins involved in ET reactions, the so called ET proteins such as ferredoxin, flavodoxin, cytochromes, etc., is that they participate in several reactions in which they have to interact with different proteins (enzymes). That requires, either that all of the enzymes present the same interface surface or that the ET protein has a distribution of amino acidic groups on its surface that is diverse enough to be able to interact with all its partners. This implies that there are complexes that make tighter binding, while others make more loose interactions. This has led to the proposal that there are complexes that exist in a single orientation, that is, there is a complex more stable than the others in which the ET reaction occurs. Others exist as dynamic ensembles with no well-defined binding sites and the ET reaction occurs during the transit from one complex to the other taking advantage of the rapidity of the reaction. There is also the possibility of forming

complexes somewhere between both models (Ubbink et al. 2003). The system that has been presented in this review offers two different models of transient complexes in ET proteins: the FNR:Fd complex is closer to the single orientation model, since the interaction between FNR and Fd shows to be quite specific. That of FNR:Fld seems to be closer to the dynamic ensemble one, since the presence of critical residues (either charged or hydrophobic) has not been probed yet. The viability of this less specific model would be possible if the reaction between the two redox centres is somehow facilitated. In the FNR:Fld we propose that this is so because the two flavin cofactors get at a very short distance once the complex is formed.

Similar requirements to those found for efficient interaction between FNR and its protein ET partners would be also required for the enzyme interaction with its coenzyme, NADP^+/H , that is, that electrostatic, hydrogen and hydrophobic interactions are the main contributors to the binding and will determine the enzyme specificity. In this context, the data here presented indicate that this is not such a simple interaction as could be expected. Thus, a step by step recognition of the coenzyme by the protein, which produces sequential rearrangements of the backbone, seems to take place. Moreover, although for the moment it has been shown that in FNR the 2'-P binding region of NADPH determines coenzyme specificity, the reported data clearly indicate that other regions of the protein, yet to be identified, might also be involved in this process. Finally, structural and biochemical data further support the existence of a ternary complex between FNR, Fd and NADP^+ during the enzyme catalytic mechanism, since the backbones modifications introduced in FNR upon coenzyme binding appear to provide additional stabilising interactions with the ET protein.

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