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Protein-protein interaction in electron transfer reactions: The ferredoxin/flavodoxin/ferredoxin:NADP$^+$ reductase system from *Anabaena*

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Abstract — Electron transfer reactions involving protein-protein interactions require the formation of a transient complex which brings together the two redox centres exchanging electrons. This is the case for the flavoprotein ferredoxin:NADP$^+$ reductase (FNR) from the cyanobacterium *Anabaena*, an enzyme which interacts with ferredoxin in the photosynthetic pathway to receive the electrons required for NADP$^+$ reduction. The reductase shows a concave cavity in its structure into which small proteins such as ferredoxin can fit. Flavodoxin, an FMN-containing protein that is synthesised in cyanobacteria under iron-deficient conditions, plays the same role as ferredoxin in its interaction with FNR in spite of its different structure, size and redox cofactor. There are a number of negatively charged amino acid residues on the surface of ferredoxin and flavodoxin that play a role in the electron transfer reaction with the reductase. Thus far, in only one case has charge replacement of one of the acidic residues produced an increase in the rate of electron transfer, whereas in several other cases a decrease in the rate is observed. In the most dramatic example, replacement of Glu at position 94 of *Anabaena* ferredoxin results in virtually the complete loss of ability to transfer electrons. Charge-reversal of positively charged amino acid residues in the reductase also produces strong effects on the rate of electron transfer. Several degrees of impairment have been observed, the most significant involving a positively charged Lys at position 75 which appears to be essential for the stability of the complex between the reductase and ferredoxin. The results presented in this paper provide a clear demonstration of the importance of electrostatic interactions on the stability of the transient complex formed during electron transfer by the proteins presently under study. © Société française de biochimie et biologie moléculaire / Elsevier, Paris

FNR / ferredoxin / flavodoxin / protein-protein interaction

1. Introduction

Electron transfer reactions involving protein-protein interactions require the formation of a transient complex which brings together the two redox centres exchanging electrons. During oxygenic photosynthesis four electrons are removed from two water molecules, yielding molecular oxygen and protons. The two high potential electrons removed from water are used to reduce one NADP$^+$ molecule. In the course of the oxidation-reduction reactions involved in electron transfer from water to NADP$^+$, enough energy is also liberated to synthesise one ATP molecule. Ferredoxin:NADP$^+$ reductase (FNR) catalyses the transfer of two electrons from reduced ferredoxin (Fd) to NADP$^+$ [1]. This enzyme contains a non-covalently bound FAD with a midpoint redox potential of –344 mV (pH 7.0) [2]. Plant and cyanobacterial ferredoxins are small globular proteins (11 kDa), which contain one [2Fe-2S] centre which participates in reactions in which electrons are transferred at low potentials (E'o = –420 mV) [3]. Under iron-deficient conditions, certain strains of cyanobacteria and eukaryotic algae synthesise an FMN-containing flavoprotein, flavodoxin (Fld), which replaces ferredoxin in those reactions in which the iron-protein is involved [3]. Its participation in photosynthesis is, then, to substitute for Fd in the transfer of one electron from PSI to FNR, for which it uses the half reaction in which the protein oscillates between the semiquinone and hydroquinone forms, at a midpoint redox potential of –436 mV (at pH 7) [2]. The reaction in which these three proteins are involved requires the formation of a transient complex between Fd and FNR, or Fld and FNR, so that electron

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Abbreviations: FNR, ferredoxin:NADP$^+$ reductase; FNR_{ox}, FNR in the oxidised state; FNR_{rd}, FNR in the reduced state; FNR_{sq}, FNR in the semiquinone state; 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; Fld_{sq}, Fld in the semiquinone state; IPTG, isopropyl-β-D-thiogalactoside; dRf, 5-deazariboflavin; dRfH, semiquinone form of dRf; et, electron transfer.

transfer between the two proteins can occur. It is now well established that the rate of interprotein electron transfer is modulated by parameters that include: a) a thermodynamic term that takes into consideration the difference in redox potentials between the prosthetic groups which are participating in the reaction; b) the distance between the two centres that exchange electrons; and c) what is called the 'reorganisation energy', a term that refers to the change in geometry that protein and solvent molecules undergo during the formation of the transition state of the reaction. This implies that the rate of electron transfer would depend on the structural characteristics of the protein-protein interface during the electron transfer step, as well as distance, relative orientation and redox potential differences between the two centres.

The three dimensional structures of *Anabaena* FNR [4], Fd [5] and Fld [6] have been determined at high resolution, providing invaluable information for the study of this interesting problem. The three-dimensional structure of *Anabaena* FNR resembles that reported for the spinach enzyme [7], consisting of two domains. One of these binds FAD, and is made up of a scaffold of six antiparallel strands arranged in two perpendicular β-sheets, the bottom of which is capped by a short α-helix and a long loop [4]. The $NADP^+$ binding domain consists of a core of five parallel β-strands surrounded by seven α-helices. This arrangement corresponds to a variant of the Rossman fold typical of dinucleotide binding proteins [8]. Moreover, the three-dimensional structure of this enzyme has been proposed to be the prototype of a large family of flavin-dependent oxidoreductases that function as transducers between nicotinamide dinucleotides (two-electron carriers) and one-electron carriers [7, 9, 10]. The FNR molecule has a very noticeable concave cavity, comprising both the FAD and $NADP^+$ binding domains, where the Fd (and Fld) is proposed to bind [4, 11, 12]. The FAD molecule is located in the centre of this cavity with its dimethylbenzene ring, the part of the cofactor molecule through which the electrons must be exchanged, pointing towards the solvent. Chemical cross-linking of FNR to Fd [13], chemical modification [12, 14–16] and site-directed mutagenesis [17, 18] experiments have identified, in both the spinach and the *Anabaena* enzymes, a number of important arginine and lysine residues in the area where the Fd interaction is proposed to occur [4, 11].

In recent years, a large amount of structural, kinetic and electrochemical data that demonstrate the crucial role that certain amino acid residues in ferredoxin play in the reaction with FNR have been reported [19–23]. Similar studies are now in progress to study the role of residues at the FNR surface [18, 24]. The data presented herein summarises our work concerning the role of acidic amino acid residues in *Anabaena* Fd, or Fld, and basic residues in *Anabaena* FNR at the protein-protein docking interfaces.

2. Materials and methods

2.1. Oligonucleotide-directed mutagenesis

Mutants of recombinant *Anabaena* PCC 7120 Fd were made using the Transformer™ site-directed mutagenesis kit from Clontech and a construct of the *pet*F gene, pAn662 [25], cloned into the pIBI25 vector (International Biotechnologies Inc., New Haven, CT, USA) as template. *E. coli* strain JM109 was transformed with this plasmid for protein expression. Mutants of recombinant *Anabaena* PCC 7119 FNR were produced using a construct of the *pet*H gene which had been previously cloned into the expression vector pTrc99a [26]. Amino acid substitutions were also carried out using the Transformer site-directed mutagenesis kit from Clontech (Palo Alto, CA, USA). The constructs containing the mutated FNR gene were used to transform the *E. coli* PC 0225 strain [27]. Mutations were verified by DNA sequence analysis.

2.2. Purification of proteins

Anabaena PCC 7119 wild type and mutant FNR forms were purified from IPTG induced cultures as previously described [24, 27]. *Anabaena* PCC 7120 wild type and mutant ferredoxins were purified as previously described [19]. Recombinant flavodoxin from *Anabaena* PCC 7119 was prepared as described [28]. UV-visible absorption spectra and SDS-PAGE electrophoresis were used as purity criteria.

2.3. Binding constants for the Fd_{ox}:FNR_{ox} complexes

Dissociation constants, binding energies and extinction coefficients of the complexes between oxidised FNR species and oxidised ferredoxin or oxidised flavodoxin were obtained as previously described [29]. Experimental data were fit to a theoretical equation for 1:1 stoichiometry by means of non-linear regression.

2.4. Laser flash photolysis measurements

The pulsed laser photolysis apparatus used to obtain transient electron transfer kinetics has been described previously [30–32], as has the photochemical reaction which initiates electron transfer [33–35]. Laser flash-induced kinetic measurements were performed at room temperature. In addition to protein, samples also contained 1 mM EDTA and 95–100 μM dRf in 4 mM potassium phosphate buffer, pH 7.0. When necessary, the ionic strength of the solution was adjusted to 100 mM using 5 M NaCl. Samples were made anaerobic by bubbling for 1 h with H_2O-saturated Ar gas prior to addition of protein. Binding constants for the transient Fd_{rd}:FNR_{ox} complexes were determined by fitting the laser flash photolysis data to the exact solution of the differential equation describing

a minimal (two-step) mechanism involving complex formation followed by electron transfer [36].

2.5. *Stopped-flow measurements*

Electron transfer processes between FNR and Fd, or Fld, were studied by stopped-flow methods using an Applied Photophysics SX17.MV spectrophotometer interfaced with an Acorn 5000 computer using the SX.17MV software of Applied Photophysics. Samples were made anaerobic by successive evacuation and flushing with O_2-free Ar in special tonometers which fitted the stopped-flow apparatus. All reactions were carried out in 50 mM Tris-HCl, pH 8.0, at 13 °C, and at the wavelengths appropriate to follow the reactions. The observed rate constants (k_{obs}) were calculated by fitting the data to mono- or bi-exponential processes. Reduced samples of Fd, Fld and FNR for stopped-flow were prepared by photoreduction with 5-deazariboflavin as described previously [24].

3. Results and discussion

3.1. *Arrangement of acidic residues on the surface of Anabaena ferredoxin*

Examination of a side view of the ferredoxin structure using computer graphics indicates that the iron-sulphur centre forms a protrusion in the compact protein molecule (*figure 1*). The cofactor is buried under a number of amino acid residues, mainly comprised of the two cysteines which bind one of the two iron atoms and by polar uncharged amino acids, such as Thr and Ser [5]. No charged residues are located in the immediate vicinity of the [2Fe-2S] centre. However, two distinct negative patches are located at both sides of the equatorial position as shown in *figure 1*. One of the patches is comprised of residues Asp28, Glu31, Glu32 and Asp36. The second patch of negative charge is formed by residues Asp67, Asp68 and Asp69. Both regions are well conserved among the different cyanobacterial ferredoxins [25, 37, 38]. There is also a third region of negative charge, which is conserved in vegetative cyanobacterial ferredoxins but not in the ferredoxin isozyme found in heterocyst cells [39], which consists of several amino acids located near the carboxyl terminus, Glu94, Glu95 and Glu96. This third cluster of negative charge is also located somewhat closer to the iron-sulphur centre (*figure 1*). Thus, the ferredoxin molecule is organised as a quasi-spherical molecule with the reaction site near the surface at the equator of the molecule, and negative charges asymmetrically located at the periphery of the sphere. This distribution of negative charge suggests that these patches might be involved in the orientation of the ferredoxin molecule as it approaches the reductase. Once the two molecules form a complex,

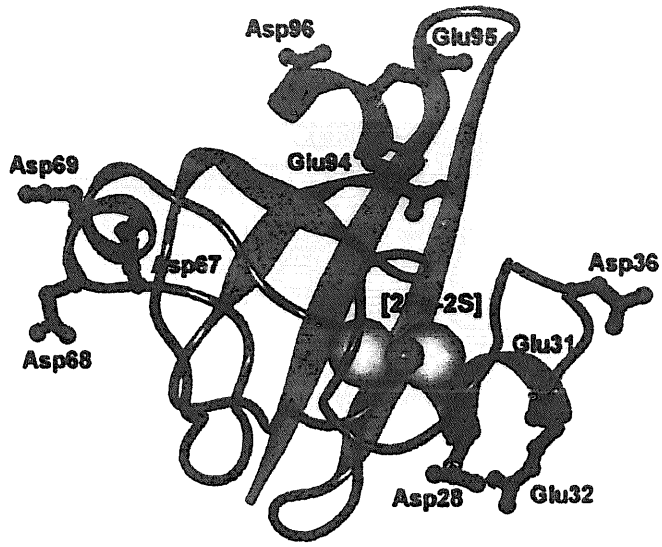

Figure 1. Schematic view of *Anabaena* ferredoxin surface in which the negative charge distribution around the [2Fe-2S] centre is indicated.

the [2Fe-2S] centre would be positioned close to the FAD centre of the reductase, ready to perform its electron transfer function. In the case of spinach ferredoxin, the presence of a molecular dipole, created by the presence of the two domains of negative potential in the molecule, with its negative end lying just above the iron-sulphur centre, has also been proposed [40]. This charge distribution has prompted us to focus on the complementary distribution of positive charges in FNR and has led to the suggestion of a number of such sites as possible candidates for involvement in the interaction with ferredoxin [18].

3.2. *Arrangement of acidic residues on the surface of Anabaena flavodoxin*

Charge distribution on the surface of *Anabaena* flavodoxin [6] follows a pattern similar to that described above for ferredoxin (*figure 2*). The FMN molecule is at one side of the protein, exposing only the dimethylbenzene ring to the solvent. Negative charges are distributed on both sides of the FMN in two patches which comprise Asp65 and Glu67 on one side, and Asp144, Glu145 and Asp146, located at the opposite side of the molecule. Other negative amino acid residues, which could also be involved in the interaction of flavodoxin with the reductase (Glu16 and Asp129) are distributed on the surface around the FMN molecule (*figure 2*). Chemical modification studies have also indicated that the region containing Asp144, Glu145, Asp146 and Asp129 must be involved in the interaction with FNR [41].

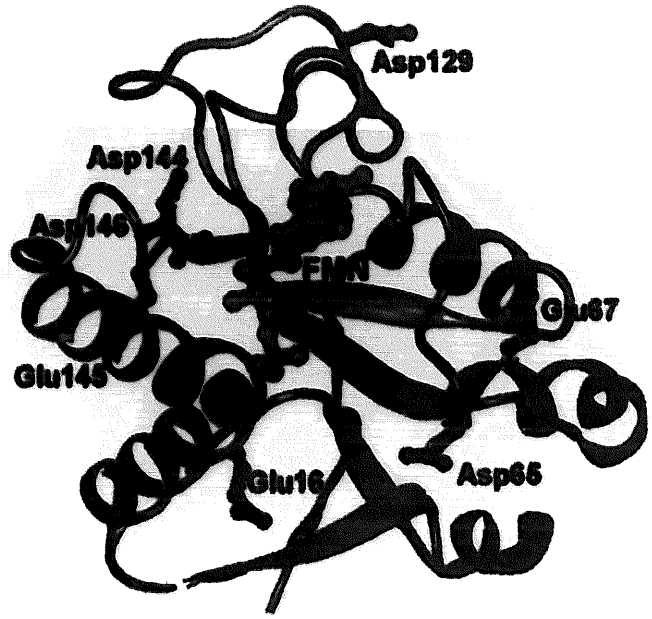

Figure 2. Schematic view of *Anabaena* flavodoxin surface in which the negative charge distribution around the FMN cofactor is indicated.

3.3. The role of negative charges in ferredoxin (and flavodoxin) in determining the rate of electron transfer with FNR

Electron transfer reactions between *Anabaena* ferredoxin (or flavodoxin) and FNR have been proposed to occur via a mechanism that involves the formation of a transient complex between the two proteins prior to the actual electron transfer process. The laser flash photolysis technique has allowed the study of such fast processes [21]. The laser flash produces the rapid reduction of ferredoxin present in a solution with FNR. The spectral changes occurring after the laser flash enable one to follow the electron transfer reactions and to determine the rate constant (k_{obs}) which, in some cases, can be measured at saturating FNR concentrations, where the rate becomes FNR concentration independent. Under these latter conditions, the reaction rate constant that is determined is due to intracomplex electron transfer (k_{et}), which includes factors such as structural rearrangement and changes in hydration of both protein and redox cofactor during transition state formation (reorganisation energy). Overall, the reaction is described by the (minimal) two-step mechanism shown in equation 1 [36]:

$$Fd_{rd} + FNR_{ox} \underset{}{\overset{K_d}{\rightleftharpoons}} [Fd_{rd}\text{-}FNR_{ox}] \xrightarrow{k_{et}} Fd_{ox} + FNR_{sq} \quad (1)$$

A similar scheme would apply when flavodoxin acts as the electron donor to FNR. It has been previously shown that laser flash photolysis of solutions containing flavodoxin and FNR resulted in the reduction of the FAD cofactor of FNR followed by a rapid electron transfer from FNR semiquinone to the flavodoxin to generate its FMN semiquinone form [42], as shown in equation (2):

$$Fld_{ox} + FNR_{sq} \underset{}{\overset{K_d}{\rightleftharpoons}} [Fld_{ox}\text{-}FNR_{sq}] \xrightarrow{k_{et}} Fld_{sq} + FNR_{ox} \quad (2)$$

In some cases saturation is not obtainable in the accessible FNR concentration range. In these situations second-order rate constants are measured, which include the complex association constant as well as k_{et}. Mutations of a number of different acidic amino acids present on the surface of the ferredoxin and flavodoxin molecules have been performed and the effect of the mutation evaluated through the determination of the kinetic parameters for the electron transfer reaction.

3.4. Arrangement of basic residues on the surface of Anabaena FNR

The FNR three-dimensional structure shows a cavity at the bottom of which is located the isoalloxazine ring of the FAD molecule (*figure 3*). Such a cavity could easily accommodate small protein molecules such as ferredoxin or flavodoxin, since the concave cavity found in the FNR complements the convex surface of either of the two electron transport proteins that interact with it. Taking into account the data available from cross-linking and chemical modification studies, using either spinach or *Anabaena* FNR [13–16], and the three-dimensional structures of the molecules, a number of basic amino acid residues are clearly distinguished on the perimeter of the aforementioned cavity which can potentially be involved in the protein-protein interaction. Again, there are two groups of charged residues which are at opposite sides of the FAD cofactor. One patch is formed by Lys72, Lys75 and Arg77, while the other is formed by Lys290, Lys293 and Lys294. Moreover, a number of other positively charged residues, Arg16, Lys138, Arg264, are distributed around the FAD cofactor forming a crown of positive charge (*figure 3*).

3.5. Charge reversal mutations in the 67–69 negative patch of Anabaena ferredoxin

The charge reversal mutants Asp67Lys, Asp68Lys and Asp69Lys, as well as the double and triple mutants at such positions, were prepared and their rates of electron transfer to FNR determined by laser flash photolysis. The hyperbolic dependence of the observed rate constants on the FNR concentration and the dissociation constants obtained for the Fd_{ox}:FNR_{ox} complexes obtained by differential spectroscopy allowed the determination of the k_{et} and the K_d values for the transient Fd_{rd}:FNR_{ox} complexes by fitting the kinetic data to the exact solution of

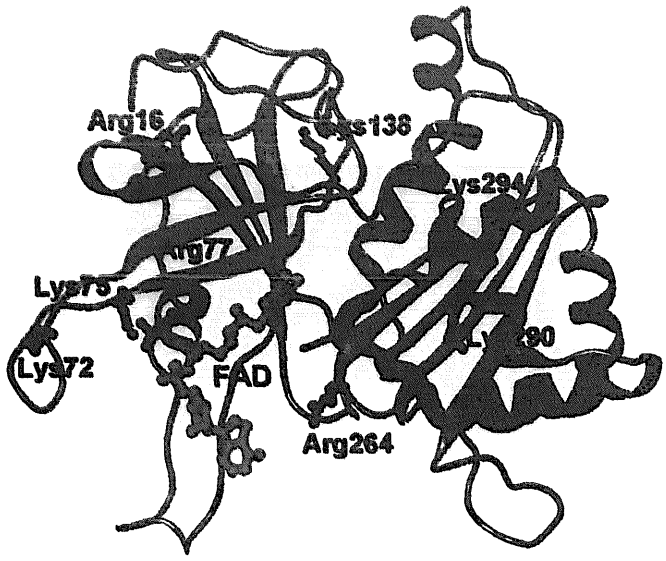

Figure 3. Schematic view of *Anabaena* FNR surface in which the positive charge distribution around the FAD cofactor is indicated.

the differential equation describing the process (equation 1). The results obtained *(table 1)* show that the charge reversal at these positions produced a slight decrease in the k_{et} values obtained at low ionic strength, except when Asp68 is replaced by a lysine. It is expected that the introduction of a positive charge on ferredoxin at a position that is involved in the interaction with its partner would produce an impairment of complex formation and, therefore, of the electron transfer ability. The behaviour of the single mutants, Asp67Lys and Asp69Lys, and the double mutant Asp67Lys/Asp69Lys are in agreement with this idea. However, the Asp68Lys Fd mutant is anomalous in that it showed increased rates of electron transfer *(table 1)*. Consistent with this, the double mutant Asp68Lys/Asp69Lys, had wild-type like activity, probably because the impairment due to the Asp69Lys mutation is offset by the anomalous increased activity of the Asp68Lys mutation. To explain these results, it has been suggested that there is a specific interaction between Asp68 and a complementary (presumably positively charged) residue on FNR which forces the proteins to assume a less favourable orientation at low ionic strengths (where electrostatic forces are stronger) during electron transfer [23]. In this context, it is interesting to note that the Asp67Lys and Asp69Lys mutants are also impaired at higher ionic strengths, whereas the Asp68Lys mutant still shows wild-type activity [23]. No correlations between complex thermodynamic stabilities (K_d) and reaction rate constants were observed *(table 1)*, indicating that the stability of the complex is not directly related to the efficiency of the electron transfer reaction. The interpretation of these results points to a model in which ferredoxin and FNR form a complex which is stabilised by electrostatic interactions. However, the fact that the binding is tight does not necessarily mean that the two protein cofactors are in the correct orientation for the efficient transfer of the electron. In fact, the ionic strength dependencies show that if the complex is too tight the mobility of ferredoxin is hindered in such a way that it cannot re-orient properly after collisional contact to produce a productive complex with FNR [31]. It can also be concluded that the role of the negative patch in the 67–69 region of ferredoxin is to produce a dipole that orients the ferredoxin molecule as it approaches FNR to start the catalytic cycle.

3.6. Charge reversal mutations in the carboxyl terminus region of Anabaena ferredoxin.

Glutamic acid residues in positions 94 and 95 in *Anabaena* ferredoxin have also been replaced by residues bearing a positive charge [19, 20]. The results obtained for these two Fd mutants were completely different. Elimination or reversal of the negative charge at position 94 results in almost complete abolition of its ability to transfer electrons to FNR, whereas the corresponding replacements at position 95 do not have any effect on the electron transfer reaction. Thus, the k_{et} for the reaction between Glu94Gln Fd and FNR was 0.34 s^{-1}, as compared to 3600 s^{-1} for the wild type Fd *(table 1)*, and the value for Glu94Lys Fd was too small to be estimated. Comparison of the second order rate constants for Glu94Gln and Glu94Lys *(table 1)* clearly shows that the removal of a negative charge at that position produces a much less reactive protein. However, the Fd_{ox}:FNR_{ox} dissociation constants for the complexes with these mutant Fds are only three-fold and six-fold lower, respectively, than that for wild-type Fd. This indicates that decreases in the stability of the complex cannot account for the drastic decrease of the electron transfer activity. Therefore, a very important role in the electron transfer reaction is indicated for the acidic residue in position 94 of Fd. That role could be related to the hydrogen bond which exists between Glu94 and Ser47. Thus, it has been found that the Ser47Ala mutant is also highly impaired in its electron transfer activity with FNR (whereas the Ser47Thr and Thr48Ala mutants are not), and that both replacements, Glu94Lys and Ser47Ala, cause a significant positive shift in the reduction potential of the iron-sulphur centre of these proteins, although this shift is not the cause of the activity changes [43]. These results point to the requirement for a highly specific protein-protein geometrical interface, i.e., the effect of making identical replacements of two contiguous residues (Glu94Lys and Glu95Lys, as well as Ser47Ala and Thr48Ala) produces completely different effects on the reactivity of ferredoxin.

Table I. Second order rate constants and corrected[a] kinetic parameters for the oxidation of reduced ferredoxins by oxidised native FNR[b].

Fd form	K_d [Fd_{ox}:FNR_{ox}] (μM)	$k \times 10^{-8}$ ($M^{-1}s^{-1}$)[c]	k_{et} (s^{-1})	K_d [Fd_{red}:FNR_{ox}] (μM)
Wild type	4.5 ± 0.6[e]	1.2 ± 0.1[d]	3600 ± 400[e]	2.2 ± 3[e]
Asp67Lys	1.8 ± 0.1[k]	0.8 ± 0.1[f]	2300 ± 300[k]	0.5 ± 0.1[k]
Asp68Lys	0.7 ± 0.2[k]	1.9 ± 0.2[d]	9000 ± 2000[k]	0.5 ± 0.1[k]
Asp69Lys	1.8 ± 0.3[k]	0.9 ± 0.1[f]	2900 ± 200[f]	0.9 ± 0.1[f]
Asp67/68Lys	4.0 ± 0.7[k]	0.4 ± 0.1[f]	2000 ± 100[k]	3.8 ± 0.2[k]
Asp68/69Lys	1.9 ± 0.3[k]	1.1 ± 0.1[d]	4400 ± 400[k]	1.2 ± 0.1[k]
Asp67/68/69Lys	n.d.[g]	0.5 ± 0.2[f]	n.d.[h]	n.d.
Glu95Lys	n.d.	1.2 ± 0.1[d]	n.d.	n.d.
Glu94Lys	26 ± 3[d]	0.00005[d]	n.d.	n.d.
Glu94Gln	13 ± 4[i]	0.00013[i]	0.34 ± 0.03[i,j]	n.d.

[a]The data were corrected for non-productive complex formation as described in [31].
[b]Experiments were carried out at an ionic strength of 12 mM except where otherwise indicated.
[c]Second order rate constants.
[d]Taken from [19].
[e]Taken from [31].
[f]Taken from [22].
[g]Complex formation could not be detected.
[h]Not determined.
[i]Taken from [21].
[j]This experiment was carried out at 100 mM ionic strength.
[k]Taken from [23].

3.7. Mutation of negatively charged amino acid residues on the surface of Anabaena flavodoxin

Several negatively charged residues at the surface of *Anabaena* flavodoxin have previously been replaced by neutral residues: Glu61Gln, Glu67Ala, Asp100Gln, Asp126Ala and Glu145Ala [44]. Only relatively small differences in the rates of electron transfer between FNR_{ox} and these mutated flavodoxins were observed when compared to the wild-type flavodoxin. These changes probably reflect alterations in the mutual orientations of the two proteins within a preformed complex. The largest decreases in reactivity were observed for the Asp126Ala and Glu67Ala flavodoxin mutants. With the limited amount of information thus far available for this protein it is not possible to conclude if these charged amino acid residues on the surface of *Anabaena* flavodoxin play a significant role in the electron transfer reaction. The small effects observed may be a consequence of the fact that no charge reversals have been made in these mutants, but only charge neutralisations.

3.8. Charge reversal of basic amino acid residues on the surface of Anabaena FNR

Some of the residues forming the positive patches around the FAD of *Anabaena* FNR have been replaced, using site-directed mutagenesis, by both negatively charged and neutral amino acids [18]. The ability of the corresponding mutated proteins to transfer electrons with both Fd and Fld has been studied using steady-state and rapid kinetic techniques, and the stability of their complexes with FNR has been determined. For the Arg77Glu and Arg77Gln replacements, no active mutants that properly bind FAD have been obtained [18] and thus the study of the involvement of Arg77 in the interaction of FNR with ferredoxin or flavodoxin has not been possible. These results indicate the important function of the guanidinium group of Arg77 in the electrostatic stabilisation of the pyrophosphate group of the FAD cofactor [4]. The steady-state kinetic characterisation of the Arg16Glu, Lys72Glu, Lys75Glu, Lys138Glu, Arg264Glu, Lys290Glu and Lys294Glu FNR mutants revealed three different types of behaviour in those reactions in which complex formation and electron transfer to/from ferredoxin were involved, whereas no major changes were detected for any of the mutants with respect to the wild type FNR in their reactivity with $NADP^+$ /NADPH [18]. These three types of FNR mutants, distinguished by their behaviour toward Fd, were also found when the laser flash photolysis technique was applied to the study of the electron transfer reaction from reduced ferredoxin to the different FNR_{ox} mutants (*table II*). While replacement of amino acid residues 138, 264, 290 and 294 by negatively charged amino acids did not markedly affect the rate constants for electron transfer from Fd_{red}, the introduction of a negative charge at positions 16 and 72 produced substantial decreases in the electron transfer rate which could be observed by the laser photolysis technique (see the second order rate constants in *table II*). The third type of

Table II. Fast kinetic parameters of electron transfer from reduced Fd to oxidised wild type and mutant FNRs studied by laser flash photolysis[a].

FNR form	K_d [Fd_{ox}:FNR_{ox}] (μM)	$k \times 10^{-8}$ ($M^{-1}s^{-1}$)[d]	k_{et} (s^{-1})	K_d [Fd_{red}:FNR_{ox}] (μM)
Wild type[b]	0.3 ± 0.1[f]	7[c]	3600 ± 400	2.2 ± 0.3
Arg16Glu	120 ± 20	1	n.d.[b]	n.d.[b]
Lys72Glu	50 ± 15	2	n.d.[b]	n.d.[b]
Lys75Glu	n.d.[c]	0.05[g]	> 165	n.d.[c]
Lys138Glu	4.1 ± 0.8	10	3700 ± 400	1.8 ± 0.2
Arg264Glu	3.3 ± 1.1	13	3800 ± 500	1.3 ± 0.2
Lys290Glu	4.0 ± 1.6	9	3500 ± 600	1.9 ± 0.3
Lys294Glu	7.2 ± 1.3	9	4800 ± 300	2.9 ± 0.2

[a]In addition to protein, solutions also contained 0.1 mM dRf and 1 mM EDTA in 4 mM potassium phosphate, pH 7.0 (μ = 12 mM).
[b]Not able to be accurately determined.
[c]Complex formation was not detected.
[d]Estimated from the initial slopes of the k_{obs} vs. [FNR] curves after correction for pre-formed complex (see [31] for details).
[e]Estimated as in [31].
[f]Taken from [31].
[g]This value was not corrected for pre-existing complex since K_d could not be measured.

behaviour is that shown by Lys75Glu FNR, which is almost completely impaired in its electron transfer reaction with Fd. The dissociation constants measured for the complexes formed between the Fd mutants and the reductase *(table II)* reflect the involvement of these amino acids in the stabilisation of the complexes. This is especially true for the Lys75Glu mutant for which the binding constant for the complex was too weak to be measured. These results provide strong evidence that all of the mutated residues in *Anabaena* FNR are involved in Fd binding, with Lys75 being most important, Arg16 and Lys72 being somewhat less important, and the other four residues far less important for the binding process. Further protein engineering on Lys75 has also been carried out in order to investigate its role in the electron transfer interaction, and the arginine, glutamine and serine mutants have been prepared. Laser flash photolysis experiments in which reduction of the different FNR mutants at position Lys75 by Fd_{red} at I = 100 mM indicates that the Lys75Arg mutant *(table III)* behaves similarly to the wild-type FNR, whereas saturation could not be detected for the Lys75Gln and Lys75Ser FNR forms to allow k_{et} and K_d for the Fd_{red}:FNR_{ox} complex to be calculated. The observed second order rate constants for the electron transfer processes with the different FNR forms show that Lys75Gln and Lys75Ser are kinetically quite similar to each other, and that Lys75Glu is severely impaired in its electron transfer interaction with Fd.

Table III. Kinetic parameters for the reduction of the different Lys75 FNR mutants studied by laser flash photolysis[a]

FNR form	K_d [Fd_{ox}:FNR_{ox}] (μM)	$k \times 10^{-8}$ ($M^{-1}s^{-1}$)[c]	k_{et} (s^{-1})	K_d [Fd_{red}:FNR_{ox}] (μM)
Wild type[b]	3.3 ± 0.6	2.7 ± 0.4	5500 ± 400[d,e]	1.7 ± 0.1[d,e]
Lys75Arg	4.8 ± 0.5	1.5 ± 0.2	4900 ± 600[d]	3.0 ± 0.4[d]
Lys75Gln	380 ± 20[f]	0.38 ± 0.11	-	-
Lys75Ser	200 ± 60[f]	0.56 ± 0.02	-	-
Lys75Glu	n.d.[g]	0.0082 ± 0.0003	-	-

[a]Deaerated solutions also contained 100 μM 5-deazariboflavin and 1 mM EDTA in 4 mM potassium phosphate buffer, pH 7.0. The ionic strength was adjusted to 100 mM using 5 M NaCl.
[b]Taken from [24].
[c]Second order rate constants were estimated for wild type and Lys75Arg from initial slopes of the hyperbolic dependence of k_{obs} vs. [FNR] curves (not corrected for the concentration of pre-formed complex). Second order rate constants for the other mutants were measured directly from the linear plots.
[d]Corrected for the concentration of preformed complex.
[e]Taken from [31].
[f]Binding was too weak to observe saturation in the experimentally accessible protein concentration range. Therefore, K_d was determined from difference spectra, assuming that the difference extintion coefficient for the complex was the same as that Δε calculated for the complexes of Lys75Arg and wild type FNR with wild type Fd (Δε value is 2000 ± 300 M^{-1} cm^{-1}).
[g]The spectral perturbation due to binding was too weak to be measured for this mutant.

Table IV. Fast kinetic parameters for electron transfer reactions of wild type and charged-reversal mutant FNR forms studied by stopped-flow[a].

	k_{obs} (s^{-1}) *for the mixing of* FNR_{ox} *with*		k_{obs} (s^{-1}) *for the mixing of* FNR_{rd} *with*	
FNR form	[c]Fd_{rd}	[d]Fld_{rd}	[c]Fd_{ox}	[c]Fld_{ox}
Wild type[b]	n.d.[e]	n.d.[e]	n.d.[e]	2.5 ± 1.0
	250 ± 30			1.0 ± 1.0
Arg264Glu	n.d.[e]	14.6 ± 0.4	>200	1 ± 0.2
	161 ± 10	3.1 ± 0.2		0.75 ± 0.5
Lys75Glu	2.7 ± 0.4	0.52 ± 0.06	< 0.0011	0.11 ± 0.02
	0.50 ± 0.05	0.08 ± 0.02		0.018 ± 0.001

[a]The samples were mixed in the stopped-flow cell at the indicated redox states. T = 13 °C. All samples were prepared in 50 mM Tris-HCl, pH 8.0.
[b]Data from [24].
[c]Reaction followed at 507 nm.
[d]Reaction followed at 600 nm.
[e]Reaction occurred within the dead time of the instrument.

We have previously shown that kinetic data for the electron transfer reactions from FNR_{rd} to Fd_{ox} or Fld_{ox}, and from either Fd_{rd} or Fld_{rd} to FNR_{ox}, can be followed using anaerobic stopped-flow techniques [24]. Although this technique is not appropriate for the determination of the kinetic parameters of some of the reactions involving the wild type proteins, we have shown that it can provide important information for those mutants with altered properties either with regard to complex formation or electron transfer processes between FNR and its protein partners. The study of such reactions for the Lys75Glu and Arg264Glu FNR charge-reversal mutants *(table IV)* shows different types of behaviour for both mutants, either with Fd or Fld, that are in agreement with those found in the above mentioned steady-state and laser flash photolysis studies. As shown by laser flash photolysis *(table II)*, reduction of FNR by Fd_{rd} is greatly affected by the introduction of a negative charge at position 75, whereas charge reversal at position 264 results in wild type-like reactivity. Similar results were obtained for the two corresponding reactions when Fld is the redox partner *(table IV)*. These results indicate that mutation at these positions has similar effects on both electron transfer proteins, ferredoxin and flavodoxin, and thus provide evidence that ferredoxin and flavodoxin both must interact with FNR at the same region of the reductase. Furthermore, it is quite evident that different amino acid residues in FNR are involved to different extents in the interaction with both ferredoxin and flavodoxin. All of these data demonstrate that the charge-reversal FNR mutations which are located at the FAD-binding domain (Arg16Glu, Lys72Glu and Lys75Glu) *(figure 3)* cause dramatically altered electron transfer properties with both protein redox partners, whereas those mutations situated in the $NADP^+$-binding domain (Lys138Glu, Arg264Glu, Lys290Glu and Lys294Glu) cause considerably less impairment in the electron transfer processes to Fd and Fld.

The interaction between redox partners participating in electron transfer reactions has been the subject of study in several biological systems. In the photosynthetic electron transfer chain it was shown that interactions between acidic patches in plastocyanin with positive charges in cytochrome *f* contribute to the stabilisation of the complex [45]. Very recently, the dynamic structure of the transient complex between these two proteins has been elucidated and electrostatic interactions are proposed to 'guide' the partners into the optimal position for electron transfer [46]. Several other reports also indicate that electrostatic interactions produce the stabilisation of electron transfer complexes in biological systems. They include the interaction between mitochondrial ferredoxin and ferredoxin reductase [47], cytochrome *c* and cytochrome *c* peroxidase [48], cytochrome P-450 and flavodoxin [49], or [4Fe-4S] ferredoxin and nitrogenase [50]. According to this, it is tempting to propose that the effects on the rate of electron transfer that we have observed in the Fd-FNR system upon replacement of charged amino acids on the protein surfaces could also be the case for other protein-protein electron transfer reactions.

4. Conclusions

The results presented in this study have assessed the importance of electrostatic interactions in the formation of protein-protein complexes and subsequent electron transfer in a physiologically relevant redox system. It has been clearly demonstrated that an exceptionally high degree of specificity exists at the interface of the intermediate complex between reduced Fd and oxidised FNR, and that the mutual orientation between the redox cofactors is a crucial parameter in determining electron transfer rates. Furthermore, some of the mutated residues are shown to

be essential for achieving the correct docking orientation, while others are only moderately involved. Moreover, evidence that ferredoxin and flavodoxin must both interact with FNR at the same region of the reductase has also been shown. The combination of site-directed mutagenesis of *Anabaena* FNR and stopped-flow techniques have allowed fast kinetic studies of electron transfer between Fld and FNR mutant forms which were too fast to be followed for the wild type enzyme. Although the results obtained thus far are not sufficient to unambiguously determine the structure of the intermediate complex, we are hopeful that additional studies will permit this to be accomplished.

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