Molecular recognition in protein complexes involved in electron transfer
C. Gómez-Moreno*, M. Martínez-Julvez*, M. F. Fillat*, J. K. Hurley† and G. Tollint†
*Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, Spain, and
†Department of Biochemistry, University of Arizona, Tucson, AZ, U.S.A.

Introduction
Electron transfer reactions involving protein complexes are important in many biological processes, including photosynthesis, respiration, nitrogen fixation and steroid synthesis. The interprotein electron transfer reaction occurs either by orbital overlap or through a number of chemical bonds in the polypeptide chain that act as pathways for the electrons to communicate with the two redox centres. Flavoproteins are important components of the electron transfer chains which participate in many processes in all kinds of organisms. They all have a flavin group tightly bound to the polypeptide chain, which gives the bright yellow colour characteristic of this type of proteins. In many flavoproteins the flavin group can accommodate the exchange of either one or two electrons during the redox reaction because the oxidized, semiquinone and fully reduced forms are stable. Free flavins are stable only in the fully oxidized and fully reduced states.

Iron-containing proteins, such as cytochromes of the different classes and iron–sulphur proteins, are also present in these pathways. Although some of these proteins have more than one iron atom in the molecule, they only exchange one electron. Reactions in which reducing equivalents are transferred from (or to) pyridine nucleotide cofactors (typical two-electron transferring compounds) to iron proteins (which can accommodate only one electron) necessarily require the mediation of a flavoprotein to split the two-electron transferring reaction into two one-electron reactions. Transient complexes between flavoproteins, iron–sulphur proteins and cytochromes are, thus, the material of study of these type of reactions.

Some of the basic questions concerning the mechanism of these reactions are under intense investigation by different groups and include the following:

1. What is the nature of the forces maintaining the functionally important complex for electron transfer? Are only charge pairs important or are there also hydrogen bonds and hydrophobic interactions?

2. What is the effect of the distance between the two groups exchanging electrons? What effect has the relative orientation?

3. How does the difference in redox potential affect the rate of electron transfer?

4. Are there fixed pathways for the electrons transferred and how do they contribute to the rate of electron transfer?

Many of the questions raised here are not fully understood in biological systems although there is renewed interest in those systems in which the crystal structure of components has been revealed.

One of the first systems used to study interprotein electron transfer was the yeast cytochrome c peroxidase–cytochrome c complex, because the three-dimensional structure of the two individual components was known early and molecular biology techniques allowing the performance of site-directed mutagenesis were available at the same time. The crystal structure of a complex containing the two proteins has become recently available [1], providing a powerful tool for the analysis of this system. Nevertheless, the results of kinetic experiments using the wild-type and mutant proteins suggest that the functional complex has a different arrangement from the proposed complex structure [2]. This raises another important question with respect to the mechanism of electron transfer in protein complexes: the complex revealed by X-ray diffraction techniques is probably the most stable one that two-electron transferring proteins can form. But it has to be proven that it is the functional one and that it represents the true intermediate in the reaction.

Electron transfer between ferredoxin and ferredoxin–NADP+ reductase
Ferredoxin/ferredoxin–NADP+ reductase (FNR) is a naturally occurring protein–protein electron transfer system that is involved in the production of NADPH by reducing equivalents generated by photosystem I during the light phase of photosynthesis. FNR from the cyanobacterium Anabaena PCC 7119 is isolated as a 36-kDa protein with an FAD group non-covalently bound to the polypeptide chain and showing a mid-point
Anabaena, Escherichia coli, Julvez, J.

...point redox potential of -430 mV, which is photoreduction, which is the physiological reaction yielding NADPH and which also requires plant-type [2Fe-2S] ferredoxins. It has an... amino acid residues been resolved at high resolution and it was found that a large number of acidic amino acid residues... to that of the spinach enzyme, which was recently described [5]. Essentially it has two different domains, one in which the FAD binds and a second that is the site of binding of NADPH. Examination of the structure reveals the existence of a wide concave cavity covering the two domains, which is where ferredoxin is proposed to bind the enzyme [6]. Unexpectedly, it has recently been reported that the complete nucleotide sequence of the petH gene, which encodes FNR in Anabaena PCC 7119, is 136 amino acids longer than the enzyme obtained after purification to homogeneity [7]. Similar results were described previously in Synechococcus sp. PCC 7002, another cyanobacterium [8]. Further studies, both in Synechococcus and in Anabaena, have demonstrated that a 49-kDa protein can be isolated from the cyanobacteria or from recombinant E. coli cells in which the petH gene has been cloned and expressed and that this long protein is rapidly processed to yield the 36-kDa protein which is believed to be the one that is functionally significant (M. Martinez-Julvez, J. K. Hurley, G. Tollin, C. Gomez-Moreno and M. F. Fillat, unpublished work). As mentioned above, FNR from Anabaena has been cloned and can be expressed with high yields in Escherichia coli, allowing the performance of site-directed mutagenesis studies with this enzyme [9].

Ferredoxin from Anabaena belongs to the plant-type [2Fe–2S] ferredoxins. It has an 11-kDa polypeptide chain and a very low mid-point redox potential of ~430 mV, which is characteristic to this type of protein [10]. The structure of Anabaena ferredoxin has recently been resolved at high resolution and it was found that a large number of acidic amino acid residues reside in the surface of the protein [11]. Ferredoxin–NADP+ reductase can be assayed with different enzymatic assays [12]: (i) the diaphorase activity assay, in which the transfer of electrons from NADPH to an artificial electron acceptor, such as dichloro-phenol indophenol (DCPIP) is determined; (ii) the NADPH–cytochrome c reductase activity assay, in which FNR transfers electrons from NADPH to cytochrome c through the mediation of ferredoxin; (iii) measurement of chloroplast-dependent NADP+ photoreduction, which is the physiological reaction yielding NADPH and which also requires the presence of ferredoxin in the assay mixture; and, finally, (iv) assay of the transhydrogenase reaction, during which FNR exchanges electrons between NADPH and NAD+ and vice versa.

Previous chemical modification studies with FNR have indicated the requirement of specific amino acid residues for efficient electron transfer between FNR and ferredoxin [13,14]. Thus, treatment of FNR with phenylglyoxal indicated that Arg-224 and Arg-233 are involved in the interaction with the pyridine nucleotide, whereas Arg-77 seems to be a key residue in the interaction with ferredoxin. Similarly, modification of FNR with pyridoxal 5’-phosphate indicated that Lys-53 and Lys-294 could be involved in the interaction of FNR with ferredoxin. Further rapid kinetic studies using the laser flash photolysis technique have indicated that chemical modification of the specific residues involved in the interaction with the substrates of FNR produces very dramatic changes in the transient kinetics of the electron transfer reactions between the proteins, suggesting the involvement of these amino acids in the mechanism of complex formation and in the intracomplex redox process [15].

Reduction of FNR by wild-type and mutant ferredoxins

Site-directed mutagenesis studies with Anabaena ferredoxin have clearly indicated the involvement of two specific amino acid residues of this protein in the electron transfer reaction with FNR [16–18]. E94 and F65 have been found to be essential for the electron exchange reaction between the two proteins (see Figure 1A). The reason for the absolute requirement of these residues has not yet been clarified. It cannot be stated that the 50000-fold difference in the rate of electron transfer between the mutant ferredoxin and FNR is due to the weakness of the complex formed since the measurement of the binding constant of the complex reveals only 2- to 3-fold differences with respect to the wild-type protein. Moreover, the involvement of charged amino acid residues in the formation of a stabilizing ionic pair is reasonable, but the implication of the aromatic residue F65 in a strongly stabilizing effect is not well documented. Other possibilities exist such as that these amino acids are acting as chemical connections between the two redox groups involved or simply modifying the environment of the iron–sulphur centre such as to change its physicochemical properties.
Electron transfer between ferredoxin and wild-type and mutant FNRs
In order to clarify this point, we have performed a series of experiments aimed at the preparation of FNR mutants in which those amino acids residues believed to be involved in the interaction of the enzyme with ferredoxin, which are all positively charged ones, were replaced by others with negative charges. The decision for choosing these specific amino acids was made on the basis of modification studies, as well as by close examination of the three-dimensional structure of the enzyme. In this latter case the presence of a crown of positively charged amino acid residues is quite obvious in the concave cavity where ferredoxin is supposed to bind. Most of these residues had been proposed by Karplus and Bruns [19] as possible candidates for the interaction between the spinach enzyme and ferredoxin.

The following FNR mutants were prepared and purified (C. Gómez-Moreno, M. Martínéz-Julve, M. F. Fillat, J. K. Hurley and G. Tollin, unpublished work): K-75E, K-138E, K-290E and K-294E (see Figure 1B). All the mutant proteins showed similar UV–VIS spectra to the wild type, indicating that no major structural changes had taken place during the substitution.

Steady-state kinetic experiments
The enzymatic activities of these mutants were measured and compared with those of the wild-type protein. The turnover numbers obtained for the diaphorase activity (Figure 2A) indicated that
only the K-75E mutant had significantly lower values, since its activity is reduced to approximately 50% of that of the wild type. All the other mutants showed similar values. The turnover numbers corresponding to the NADPH–cytochrome c reductase activity (Figure 2B) showed drastic changes. Similar, or even higher values, were obtained with some mutants. The concentration of ferredoxin required in these reactions using FNR mutants was 10- to 30-fold higher than in the case of the wild type. The results for K-75E mutant are especially relevant since only some 2% of the activity was observed with respect to the wild-type protein. These results indicate that all the mutants prepared have partially impaired the interaction with ferredoxin, which is required for the cytochrome c reductase activity. The affinity for NADPH does not seem to have changed much since the effect on the diaphorase activity was minor in all cases. The case of K-75E seems to be somewhat different since the close location of the residue to the FAD group in FNR could have a more drastic effect on the properties of the flavin group taking part in the reaction. It could also be affecting the access of the dye to the active centre of the enzyme, even if the interaction of this artificial substrate with the enzyme is less specific than that of ferredoxin.

Rapid kinetic experiments
Laser flash photolysis experiments were performed with the FNR mutants to obtain direct values of the rate constants of the intermolecular electron transfer between ferredoxin and FNR mutants. Figure 3 shows that the rate constants for reduction of FNR mutants K-138E, E-290E
and K-294E by ferredoxin are all in the same range. These rates are also significantly higher than those for the reduction of wild-type FNR by ferredoxin. These results indicate that, at the low ionic strength measured, ferredoxin and FNR mutants can form complexes that are efficient in the electron transfer reaction. This is supported by the data presented above (Figure 2B) for the steady-state kinetic measurements with higher NADPH-cytochrome c reductase activity being obtained with these mutants, provided that sufficient ferredoxin was present.

The nature of the intermediate complex between ferredoxin and wild-type and mutant FNRs seems to be different since, at higher ionic strengths, the rates for the wild-type FNR increase to reach a maximum and then decrease, while those of the mutants show lower rates at increasing salt concentrations (data not shown).

The rapid kinetic data show, as occurred with the steady-state experiments, that the behaviour of the K-75E mutant is different since very low rate constants are obtained. This indicates that mutant K-75E is highly impaired for the electron transfer reaction with ferredoxin. Work is under way to characterize this mutant in order to reveal the role of residue K-75 in the electron transfer reaction supported by FNR.

**Conclusions**

We conclude that the electron transfer process between the two physiological partners ferredoxin and FNR is strongly dependent on the presence on the ferredoxin surface of a reduced number of amino acids. The negative charge in the residue in position 94 in ferredoxin is absolutely required for electron transfer, as is the presence of an aromatic residue at position 65. The actual role of residues in these positions is not clear at present. Although the involvement of a charged pair between the acidic residue at position 94 in ferredoxin with another basic residue in FNR would be possible, other interpretations could be drawn, such as, for instance, orbital overlap between the FAD in FNR and the iron-sulphur centre in ferredoxin occurs but a negative charge and, perhaps, an aromatic group carrying π-electrons in certain position helps to achieve the geometry and physicochemical properties of the Fe–S centre necessary for electron transfer.

With respect to the enzyme FNR we find two types of residues present in the surface on interaction with ferredoxin. Some of these are not essential for the electron transfer reaction but increase the affinity of the enzyme for the substrate ferredoxin (K-138, K-290 and K-294). K75 plays a more important role in the electron transfer process between the two proteins since its replacement results in the complete loss of ferredoxin-dependent activities.

Taken together, these results indicate a high degree of specialization of the two proteins involved in electron transfer since the replacement of some amino acid residues in both proteins has a moderate effect on the rate of electron transfer, whereas others are critical for that reaction. The role of these critical amino acid residues in protein–protein electron transfer is still unclear and is the focus of present investigations.

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**References**

NMR studies of flavoproteins

H. Rüterjans*, G. Fleischmann*, M. Knauf*, F. Lühr*, M. Blümel*, F. Lederer†, S. G. Mayhew‡ and F. Müller§

*Institute of Biophysical Chemistry, Johann Wolfgang Goethe-University, Frankfurt/Main, Germany, †Clinique Néphrologique, Hôpital Necker, Paris, France, ‡Department of Biochemistry, University College Dublin, Dublin, Republic of Ireland, and §Central Development Toxicology, Sandoz Agro Ltd, Basel, Switzerland

Introduction

In previous studies it has been shown that the chemical shifts of some of the $^{15}$N and $^{13}$C resonances of free flavin depend in a characteristic way on solvent polarity. Hydrogen bond formation with the solvent influences $\pi$-electron density and consequently the chemical shifts. Binding to apoflavoproteins also leads to hydrogen bond formation. This allows comparison of differences in the flavin molecule when exposed to different apoflavoproteins. The $^{15}$N and $^{13}$C chemical shift values of flavins in various flavoproteins should indicate characteristic hydrogen bonding and delocalization of electrons. Owing to this H-bond feature, it was expected that the redox properties of flavoproteins would be reflected in these chemical shift values. $^{13}$C- and $^{15}$N-NMR investigations of flavoproteins reconstituted with $^{13}$C- and $^{15}$N-labelled flavins were in partial agreement with these assumptions [1–5].

The studies of flavoproteins reconstituted with labelled FMN have been continued with flavocytochrome $b_2$ from *Saccharomyces cerevisiae*. Flavocytochrome $b_2$ is a homotetrameric enzyme with a molecular mass of 230 kDa and catalyses the oxidation of l-lactate to pyruvate in association with an electron transfer to cytochrome $c$ [6]. Each of the four subunits is composed of two domains: a haem-binding domain and an FMN-binding domain. The tertiary structure is known from X-ray analysis [7]. Flavocytochromes are of special interest since they offer a means of studying electron transfer reactions between the flavin and the haem groups in one single protein. Although this enzyme is certainly too large for a detailed study of the solution structure, the detection of the $^{15}$N and $^{13}$C resonances of the labelled FMN should be feasible.

In a further study, a refined solution structure of the *Desulfovibrio vulgaris* flavodoxin was determined for the oxidized state. This refinement was necessary for a future comparison of oxidized and two-electron reduced flavodoxin.

Results

Flavocytochrome $b_2$

Apoflavocytochrome $b_2$ has been reconstituted with FMN, chemically synthesized and isotopically labelled with $^{15}$N and $^{13}$C in distinct positions [8] (Figure 1). The chemical shifts in the resonances of these nuclei have been determined for the oxidized and two-electron reduced flavodoxin.

Oxidized form

It was possible to detect all $^{15}$N resonances of the bound FMN. A comparison with the $^{15}$N resonances of free FMN made an assignment relatively easy. Chemical shifts of $^{15}$N resonances [9] are listed in Table 1. Nitrogen atoms in heterocyclic compounds are generally classified into pyridine or $\beta$-type nitrogens and pyrrole or $x$-type nitrogens [10]. Pyridine-type nitrogens generally resonate at lower field than pyrrole-type nitrogens. Accordingly the pyridine-type atoms N(1) and N(5) resonate at lower field than the pyrrole-type atoms at N(3) and N(10). The N(1) reso-