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# Involvement of lysine-88 of spinach ferredoxin-NADP<sup>+</sup> reductase in the interaction with ferredoxin

Alessandro Aliverti<sup>a,b</sup>, Mario Ermanno Corrado<sup>a</sup>, Giuliana Zanetti<sup>a,b,\*</sup><sup>a</sup>Dipartimento di Fisiologia e Biochimica Generali, and <sup>b</sup>Centro Interuniversitario per lo Studio delle Macromolecole Informazionali, Università di Milano, I-20133 Milano, Italy

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## Abstract

A mutant of spinach ferredoxin-NADP<sup>+</sup> reductase, in which Lys-88 has been changed to glutamine, has been obtained by site-directed mutagenesis. The mutant enzyme was fully active as a diaphorase, but partially impaired in ferredoxin-dependent cytochrome *c* reductase activity. By steady-state kinetics, the  $K_m$  for ferredoxin of the K88Q enzyme was found to have increased 10-fold, whereas the  $k_{cat}$  was unaffected by the amino acid replacement. The interaction between oxidized ferredoxin and the enzyme forms was also studied by spectrofluorimetric titration:  $K_d$  values of 110 and 10 nM were determined for the mutant and wild-type proteins, respectively. These data point out the importance of a positive charge at position 88 of the reductase for the interaction with ferredoxin, confirming previous cross-linking studies.

**Key words:** Protein–protein interaction; Electrostatic interaction; Protein engineering; Ferredoxin-NADP<sup>+</sup> reductase; Ferredoxin; Flavoprotein

## 1. Introduction

Ferredoxin-NADP<sup>+</sup> reductase (FNR; EC 1.18.1.2) catalyzes the oxidation of ferredoxin (Fd) in a reaction that is thought to involve intramolecular electron transfer within a complex of the two proteins [1,2]. General interest in the FNR:Fd complex has arisen from the fact that this complex, besides being fundamental to the photosynthetic NADP<sup>+</sup> reduction, has been proposed as a representative model for many other biological electron transfer systems [3,4]. Crystallographic data are available for the single proteins [4] but not for their complex. On the basis of cross-linking studies between the reductase and ferredoxin [5,6], a model of the complex was proposed in which Fd fills in the cleft between the two domains of FNR, bringing together the C-terminal part of Fd and the 85–88 loop of the reductase [4,7]. Recently, the structure of phthalate oxygenase reductase, a single-chain protein made between a FNR-like module and a ferredoxin domain, has been solved, and attempts to align its structure with those of FNR and Fd [4] brought about a model for the FNR:Fd complex which is at

variance to the cross-linking studies [6]. Indeed, in such a model Lys-85/88 of the reductase would be far away from the Glu-92–94 cluster of Fd, residues found covalently linked through a peptide bond between the sidechain groups in the cross-linked complex [6]. However, a rotation of the docked Fd molecule of 120–180° would place the cross-linked residues closer to each other [4]. The former complex is also not completely in agreement with data from differential chemical modification of FNR and Fd in the complex [8]. According to the model proposed by De Pascalis et al. [8], the residues found cross-linked in the covalent complex [6] would be at the periphery of the binding regions.

Here we report on the properties of a FNR mutant in which Lys-88 was changed to glutamine. The data obtained indicate an involvement of this residue in the interaction of the reductase with ferredoxin, thus lending support to the first proposed model of the complex.

## 2. Materials and methods

Horse heart cytochrome *c*, INT and NADP(H) were obtained from Sigma. All other chemicals were of analytical grade. Ferredoxin was purified from spinach leaves as already described [9].

### 2.1. Oligonucleotide-directed mutagenesis

Site-directed mutagenesis of the FNR gene construct was carried out as described [10], according to the ‘gapped duplex’ method [11], using a mutagenic oligonucleotide with the sequence 5'-CAAGAATGG-ACAGCCCCATAAG-3', to replace the original AAG triplet coding for Lys-88 with the triplet CAG, which encodes glutamine. The pres-

\*Corresponding author at Dipartimento di Fisiologia e Biochimica Generali. Fax: (39) (2) 236 2451.

**Abbreviations:** FNR, ferredoxin-NADP<sup>+</sup> oxidoreductase (EC 1.18.1.2); Fd, ferredoxin; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

ence of the desired mutation and the lack of second-site mutations were confirmed by sequence analysis of the entire mutagenized gene by the chain termination method [12].

### 2.2. Expression and purification of FNR-K88Q

The fragments carrying the wild-type as well as the mutant FNR gene were re-cloned in the pMAL-c vector (New England Biolabs) to yield the constructs, pMBPFNR and pMBPFNR-K88Q, respectively, which direct the synthesis of fusion proteins where FNR is linked to the C-terminus of the *E. coli* maltose-binding protein. A substantial improvement in the level of the FNR synthesis in *E. coli* was achieved with this expression system (manuscript in preparation) in comparison to the vector previously used (pDS12/RBSII, *SphI*) [10,13]. Wild-type and mutant enzymes were purified from *E. coli* (host strain RRIAM15, harboring pMBPFNR and pMBPFNR-K88Q, respectively) according to a published purification procedure [14]. The fusion protein was cleaved into its components by a 16 h incubation at 4°C with factor Xa protease at a mass ratio of 1:500 with respect to the fusion protein, just before chromatography on the phosphocellulose column. A homogeneous preparation of FNR was then obtained by FPLC on a MonoQ column. Spectral analyses and steady-state kinetic measurements of the FNR forms were performed as already described [13].

### 2.3. Ferredoxin binding to the FNR forms

For determination of the difference absorption spectra of the complexes between the enzyme forms and Fd, FNR samples were diluted in 10 mM Tris-HCl, pH 7.7 (at 15°C), to a final concentration of ca. 20  $\mu$ M. After recording the spectrum, Fd was added at saturating concentration (25  $\mu$ M) and the final spectrum recorded. A second addition of Fd was then made to verify that no further spectral changes occurred. Difference spectra were computed by subtracting the initial spectra from the final ones, using the Hewlett-Packard 89510A general scanning software. Corrections were made for dilution due to ligand addition. The  $K_d$  values of the complexes of the wild-type and the mutant enzymes with oxidized ferredoxin were instead determined by quenching of the protein fluorescence of the reductase with ferredoxin. The FNR forms were diluted to ca. 0.5  $\mu$ M in 10 mM HEPES, pH 7.0, at 15°C, at increasing ionic strength in the range 2.4–27.4 mM, obtained by adding the proper amount of NaCl. Several additions of Fd were made and fluorescence emission spectra were recorded after each addition of Fd. Titration data were fitted to the theoretical binding equation by means of non-linear regression using the program, GraFit (Erithacus Software Ltd., UK), to optimize the values of  $K_d$ .

### 2.4. Cross-linking between FNR forms and Fd

Mixtures of 8  $\mu$ M wild-type or K88Q enzyme and 40  $\mu$ M Fd were incubated in 25 mM sodium phosphate, pH 7.0, with 5 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) in the presence of 2 mM NADP<sup>+</sup> [5]. At intervals, 1  $\mu$ l aliquots of the reaction mixtures were withdrawn, and cytochrome *c* reductase activity was measured in a 1 ml assay in the presence and absence of 8  $\mu$ M Fd.

## 3. Results

### 3.1. Expression and isolation of FNR-K88Q

The mutant FNR-K88Q was expressed by *E. coli* cells as a fusion with the maltose-binding protein at the same levels as the wild-type enzyme and it could be purified with a comparable yield. FNR-K88Q was weakly bound by the phosphocellulose matrix. However, a slight retardation in elution with respect to the unbound contaminant proteins allowed the isolation of the mutant enzyme in a highly purified form. Homogeneous enzyme could be obtained by ion-exchange FPLC. The mutant flavoprotein had an absorption spectrum identical to that of the wild-type enzyme and showed a quenched flavin fluorescence as expected (data not shown).

### 3.2. Steady-state kinetics of FNR-K88Q

FNR-K88Q was fully competent as a diaphorase using either INT or  $K_3Fe(CN)_6$  as electron acceptor (Table 1). In contrast, the mutation significantly affected the cytochrome *c* reductase activity of FNR: the mutant enzyme retained only about 60% of the activity of the wild-type protein, as measured under standard conditions at a Fd concentration of 8  $\mu$ M (Table 1). To ascertain whether the lower specific activity in the Fd-dependent reaction was due to a shift in the pH profile of the mutant enzyme, the pH dependence of the cytochrome *c* activity was studied. A change in the pH optimum for this reaction following the change of Lys-88 to glutamine could be ruled out (data not shown). Steady-state kinetic parameters for the cytochrome *c* reductase reaction were determined by varying both NADPH and Fd concentrations and by fitting the data to a 'ping-pong mechanism' model to evaluate the Michaelis constants and  $k_{cat}$ . The calculated values are reported in Table 2. Glutamine replacement of Lys-88 did not affect  $k_{cat}$  for the reaction, whereas the  $K_m$  for Fd was increased almost 10-fold.

### 3.3. Interaction with ferredoxin of FNR-K88Q

The interaction of wild-type and mutant FNRs with oxidized ferredoxin was studied to confirm what was inferred from steady-state kinetics, i.e. that the removal of the positive charge at position 88 mainly influenced the affinity of the mutant enzyme for ferredoxin. The FNR forms were titrated with oxidized Fd, and the complex formation was monitored through quenching of the FNR protein fluorescence. As shown in Fig. 1A, the same level of quenching was reached at saturating Fd concentration with both FNR forms. The  $K_d$  values obtained by curve fitting of the titration data are reported in Table 2. A difference of ca. 1.37 kcal/mol in the apparent free energy of binding of Fd to the mutant FNR with respect to the wild-type protein has been calculated through the equation:

$$\Delta(\Delta G) = RT \ln \left( \frac{K_d^{K88Q}}{K_d^{wt}} \right)$$

The value is in agreement with that calculated in the same way from the ratio of the catalytic efficiencies of the two FNR forms (i.e. 1.22 kcal/mol). The affinity for Fd of FNR-K88Q showed essentially the same ionic strength dependence as that of the wild-type enzyme

Table 1  
Specific activities of K88Q and wild-type FNRs

Enzyme form	INT <sup>a</sup> (U/FAD)	FeCN <sup>b</sup> (U/FAD)	cyt <i>c</i> <sup>c</sup> (U/FAD)
Wild-type	2,800	20,000	3,800
K88Q	2,800	20,000	2,200

<sup>a</sup>Diaphorase activity measured with INT as electron acceptor.

<sup>b</sup>Diaphorase activity measured with  $K_3Fe(CN)_6$  as electron acceptor.

<sup>c</sup>Ferredoxin-dependent cytochrome *c* reductase activity.

(Fig. 1B). The slopes obtained from the  $pK_d$  vs.  $\sqrt{\mu}$  plot for the K88Q and the wild-type FNRs are  $-9.71$  and  $-8.15$ , respectively, which compare well with the value obtained in a previous study ( $-8.9$ ) [15].

To verify that the K88Q mutation affected only the stability of the FNR:Fd complex without major effects on the binding geometry, the difference absorption spectrum of the mutant FNR induced by ferredoxin binding was recorded. The difference spectrum was very similar to that of the wild-type enzyme, indicating that Fd binding elicited essentially the same perturbations in the chromophore environment of the mutant flavoprotein as in the wild-type.

### 3.4. Cross-linking experiments

Lys-88 has been proposed to be one of the major sites on the FNR surface that become cross-linked to Fd upon treatment of the complex between the two proteins with carbodiimides [6]. Thus, it was of interest to perform cross-linking studies with FNR-K88Q, since a functional group that can be linked to a carboxylate of Fd is lacking at position 88 of this mutant form. In Fig. 2 the time-courses of cross-linking of both the K88Q and wild-type FNRs are reported by monitoring of the NADPH-cytochrome *c* reductase activity in the presence and absence of Fd. As already described [5], wild-type FNR treated with EDC in the presence of Fd acquires the capacity to catalyze electron transfer from NADPH to cytochrome *c* in the absence of free Fd. However, the cytochrome *c* reductase activity of the native cross-linked complex is about 40% lower than that of the dissociable FNR:Fd complex ([5] and Fig. 2). The cytochrome *c* reductase activity of FNR-K88Q also became progressively independent from addition of free Fd to the assay mixture during EDC incubation (Fig. 2). Thus, the K88Q mutation did not prevent Fd from being quantitatively cross-linked to the reductase. This was confirmed by SDS-PAGE analysis of the cross-linking products (data not shown). In the case of the mutant FNR, Fd cross-linking to the reductase resulted in an increase of the cytochrome *c* reductase activity rather than in an inhibition. This finding is consistent with the higher  $K_m$  for Fd shown by FNR-K88Q: the effect of the low affinity for Fd on the cytochrome *c* reduction is obviated by cross-linking the Fd to the mutant enzyme.

Table 2

Kinetic parameters for the Fd-dependent cytochrome *c* reductase reaction and dissociation constants of the complexes with Fd

Enzyme form	$V$ ( $\text{min}^{-1}$ )	$K_m^{\text{NADPH}}$ ( $\mu\text{M}$ )	$K_m^{\text{Fd}}$ ( $\mu\text{M}$ )	$K_d^{\text{Fd}}$ (nM)
Wild-type	$4,550 \pm 400$	$9.3 \pm 1.4$	$0.4 \pm 0.1$	$10 \pm 4$
K88Q	$5,000 \pm 260$	$5.5 \pm 0.5$	$3.5 \pm 0.3$	$110 \pm 12$

\* $K_d$  values determined at pH 7.0 (ionic strength = 7.4 mM) as described in section 2.

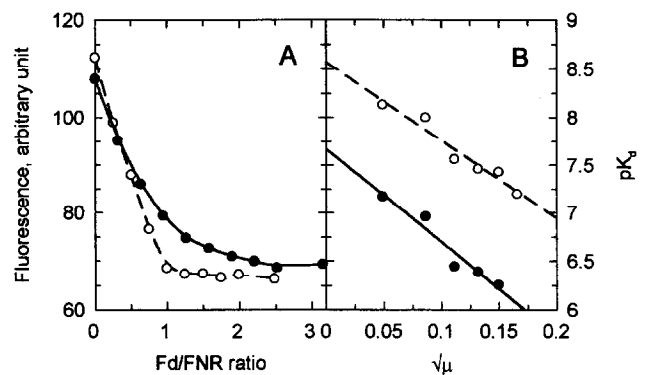


Fig. 1. Titration of K88Q and wild-type FNRs with oxidized Fd and effect of the ionic strength on the binding of Fd to the FNR forms. Experimental conditions were as described in section 2. (A) Titration with Fd of  $0.59 \mu\text{M}$  wild-type FNR ( $\circ$ ) and  $0.46 \mu\text{M}$  FNR-K88Q ( $\bullet$ ) in 10 mM HEPES, pH 7.0, containing 5 mM NaCl (7.4 mM ionic strength); (B) plot of  $-\log_{10} K_d$  vs. the square root of the ionic strength.  $K_d$  values were obtained by titrations at different NaCl concentrations.

## 4. Discussion

The mutant, FNR-K88Q, showed all the properties of the wild-type enzyme except that the interaction with ferredoxin was impaired. Lys-88 is located in an irregular loop on the surface of the protein and, generally, conservative replacements of surface residues are less likely to perturb the three-dimensional structure or stability of the protein. Mutation at position 88 resulted in a more than 10-fold decrease in the affinity of the reductase for ferredoxin, while the electron transfer rate between the protein prosthetic groups was not altered. The relatively low degree of destabilization of the FNR:Fd complex (1.37 kcal/mol with respect to the overall free energy of binding, 10.88 kcal/mol) is probably due to the rather conservative mutation made. Glutamine, indeed, could still interact through hydrogen bonding, with the Fd carboxyl group postulated to form an ion pair with the reductase Lys-88. Elimination of the amino group at this position apparently did not modify the carbodiimide-promoted cross-linking between the two proteins. It is conceivable that Lys-85 could substitute for Lys-88 in the bond formation. Moreover, it should be recalled that it was not possible to identify which of the two lysines was the residue involved in the cross-link [6]. The FNR-K88Q:Fd cross-linked complex showed a NADPH-cytochrome *c* reductase activity higher than that of the wild-type covalent complex. This may have arisen from the slightly different geometries of these cross-linked complexes. To gain further insights into the problem it will be necessary to mutagenize Lys-85 also, as well as to change Lys-88 to different residues. Nevertheless, the fact that even a rather conservative change of Lys-88 to glutamine produced a significant 10-fold decrease in the reductase affinity for ferredoxin implies that Lys-88 is functionally important in defining the fer-

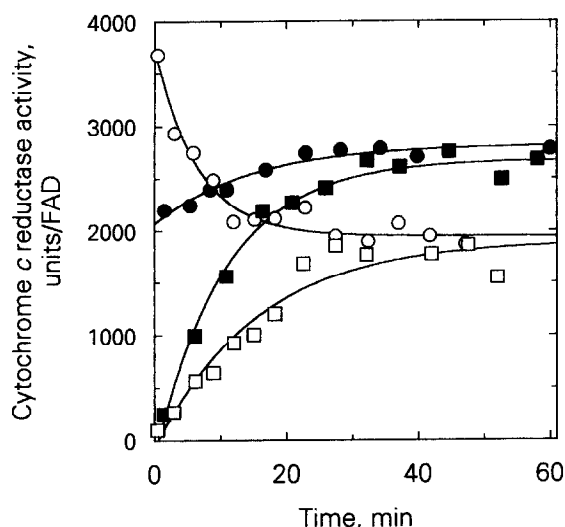


Fig. 2. Time-courses of cross-linking of Fd to wild-type and K88Q FNRs as monitored by cytochrome *c* reductase activity. Mixtures of 8  $\mu$ M wild-type or K88Q FNR and 40  $\mu$ M Fd were incubated with EDC in the presence of 2 mM NADP<sup>+</sup> under the conditions described in section 2. Aliquots of the above mixtures were diluted 1,000-fold to measure activity as stated in section 2. FNR-K88Q activity assayed in the presence (●) and in the absence (■) of added Fd to the assay; wild-type FNR activity assayed in the presence (○) and in the absence (□) of added Fd to the assay.

redoxin binding site and is not just at the periphery of the contact region between the two proteins as proposed by De Pascalis et al. [8]. Discrepancies between the two proposed models [7,8] may be reconciled if the binding of NADP<sup>+</sup> to the reductase can influence the geometry of the FNR:Fd complex. It should be recalled that the FNR:Fd cross-linked complex [5,6] was obtained in the presence of 2 mM NADP<sup>+</sup>. According to the kinetic mechanism proposed for FNR [16], the flavoprotein binds NADP<sup>+</sup> before forming a ternary complex with reduced ferredoxin, and NADP<sup>+</sup> binding greatly accelerates electron transfer from Fd to the reductase. Moreover, NADP<sup>+</sup> binding decreases the reductase affinity for oxidized Fd at least 10-fold [17]. Thus, it may well be that

the model of the FNR:Fd complex based on the cross-linking studies describes more accurately the physiological situation than that built on the basis of difference chemical modification studies performed in the absence of NADP<sup>+</sup>.

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