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Interaction of positively charged amino acid residues of recombinant, cyanobacterial ferredoxin:NADP$^+$ reductase with ferredoxin probed by site directed mutagenesis

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Abstract

The *petH* genes encoding ferredoxin:NADP$^+$ reductase (FNR) from two Anabaena species (PCC 7119 and ATCC 29413) were cloned and overexpressed in *E. coli*. Several positively charged residues (Arg, Lys) have been implicated to be involved in ferredoxin binding and electron transfer by cross-linking, chemical modification and protection experiments, and crystallographic studies. The following substitutions were introduced by site-directed mutagenesis: R153Q, K209Q, K212Q, R214Q, K275N, K430Q and K431Q in *Anabaena* 29413 FNR, and R153E, K209E, K212E, R214E, K275E, R401E, K427E, and K431E in *Anabaena* 7119 FNR. Comparison of the diaphorase activities, the specific rates of ferredoxin dependent NADP$^+$-photoreduction and cytochrome *c* reduction catalyzed by FNR showed that all these amino acid residues were required for efficient electron transfer between FNR and ferredoxin. Replacement of any one of these basic residues produced a much more pronounced effect on the cytochrome *c* reductase activity, where FNR, reduced by NADPH, acted as electron donor, than in the reduction of NADP$^+$ by photosystem I via FNR. A mutation involving the replacement of positive charge by a neutral amide produced in all cases a smaller inhibitory effect on the activity than a charge reversal mutation. In addition, it has been found that R214 was necessary for stable integration of the non covalently bound FAD-cofactor. © 1998 Elsevier Science B.V.

Keywords: Ferredoxin:NADP$^+$ reductase; Ferredoxin; Site-directed mutagenesis; Recombinant redox protein; Binding site; Cyanobacterium

1. Introduction

Ferredoxin:NADP$^+$reductase (FNR) belongs to the group of flavoproteins, which catalyze the reversible exchange of reducing equivalents between one-electron carriers and the two-electron carrying nicotinamide dinucleotides. In plants, algae and cyanobacteria FNR is involved in the transfer of electrons from photosystem I via ferredoxin to NADP$^+$. NADPH thus formed participates in a number of reductive biosynthetic processes such as CO_2-fixation and nitrogen assimilation [1]. A 1:1 complex between oxidized FNR and oxidized ferredoxin was formed, which was highly influenced by ionic strength suggesting the involvement of electrostatic interactions [2]. A catalytic cycle was proposed [3], which involves the formation of a ternary FNR–NADP(H)–ferredoxin complex. Accordingly it has been demon-

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strated that FNR binds $NADP^+$ even in the presence of ferredoxin [4]. Further support for the formation of the ternary complex was obtained when it was observed that a covalent cross-linked complex between FNR and ferredoxin (or flavodoxin) could be reduced by NADPH [5,6].

Recent crystallographic data on spinach and *Anabaena* 7119 FNR resolved to 1.7 and 1.8 Å, respectively, indicated that, in both cases, the flavoprotein consists of two domains, one involved in $NADP^+$-binding and the other in binding of the FAD cofactor [7,8]. Early studies described that NAD(P)H binding caused conformational changes in spinach FNR [9]. Crystallographic data on the complex formed by *Anabaena* FNR and $NADP^+$ revealed details of such changes which involved positively charged residues interacting with the substrate [8], but they have not yet provided, nevertheless, a real image of the geometrical relationships between the FAD-group and $NADP^+$. A mechanism involving the displacement of a tyrosine residue based on what occurs in human erythrocytes glutathione reductase [10], has been proposed for rat liver NAD(P)H:quinone reductase [11] and spinach FNR [12].

Using differential chemical modification of spinach ferredoxin, De Pascalis et al. [13] proposed that spinach FNR residues such as K33, K35, K91, R93, K304 and K305 were involved in complex formation with ferredoxin. They correspond to R153, N155 (not conserved in all FNR sequences), K212, R214, K430 and K431 in both *Anabaena* strains (the *Anabaena* residue numbering is that of the complete *petH* gene product, which corresponds to the previously described amino acid sequence by adding 137 residues [14]). The above mentioned lysine and arginine residues are arranged around a shallow cleft between the two domains of FNR [7]. In the center of this cleft, the dimethylbenzyl moiety of FAD is exposed to the solvent. The predominantly negative charge around the [2Fe–2S] cluster of ferredoxin would complement with these positive patches in FNR.

Fillat et al. [15] reported the first partial gene sequence for *petH* from a cyanobacterium encoding 304 amino acid residues. The deduced *Anabaena* 7119 FNR was 65% identical to a previously published amino acid sequence of *Spirulina platensis* [16]. Reexamination of the *petH* gene showed that the deduced complete PetH sequence of cyanobacteria contained a third domain related to CpcD, a phycocyanin linker polypeptide [14,17]. The role of CpcD is to limit the peripheral rod length variation by terminating rod length elongation [18]. A substantial portion of FNR in cyanobacteria has been shown to be associated with peripheral rods [17]. This extra domain may be important for localization of FNR close to photosystem I, but does not appear to be involved in enzyme activity as suggested by the similar kinetic behavior shown by the recombinant 49 kDa and 35–36 kDa PetH proteins [14]. It has also been suggested that the CpcD-domain of FNR folds independently [12] and is connected to the ''enzymatically active'' FNR by a hinge like structure, a possible target site of proteolysis [17].

In this paper, we present results obtained by site directed mutagenesis indicating that the interactions between recombinant, cyanobacterial FNR and ferredoxin occur through a series of conserved positively charged residues which are located around the flavin group in FNR.

2. Materials and methods

2.1. DNA manipulations

Escherichia coli strain MC 1061 [19] was used for propagation of plasmids as well as for expression of recombinant *Anabaena* 29413 FNR. *E. coli* strain PC 205 was used for expression of recombinant *Anabaena* PCC 7119 FNR. For selection of plasmids, both strains were grown in Luria-Bertani medium containing 100 μg/ml ampicillin.

All DNA manipulations were performed following standard procedures [20]. The *petH* gene from *Anabaena* 29413 was isolated from a size-fractionated gene library as a 5.1 kb *Eco*RI fragment cloned into pUC18 resulting in hybrid plasmid pFNR80. The *petH* gene from *Anabaena* 7119 was localized on a 3.5 kb *Hin*dIII fragment and cloned into pEMBL8 [15].

Overexpression of the *Anabaena* 29413 *petH* gene, site directed mutagenesis, purification and enzymatic assays were performed at the University in Bonn, whereas the same experiments with the *Anabaena* 7119 *petH* gene were carried out at the University of Zaragoza.

For overexpression of the wild-type *Anabaena* 29413 *petH*, a 2.0 kb *Hin*cII/*Mun*I DNA subfragment of pFNR80 was cloned into pUC19 (pFNRexp) with the putative *petH* promoter reading in the same direction as the *lac*-promoter. An *Xba*I/*Hin*dIII DNA subfragment carrying the *Anabaena* 7119 *petH* gene was cloned into the expression vector pTrc99A [21]. The resulting hybrid plasmid (from base 289 to the stop codon) was used to overexpress the gene fragment in *E. coli*. Directed mutagenesis by unique site elimination (USE) of both *petH* subclones was performed according to Deng and Nickoloff [22]. For this purpose a 1.5 kb *Hin*dIII/*Mun*I DNA fragment carrying the structural gene of *petH* from *Anabaena* 29413 was cloned in pUC18 without the *petH* promoter region and reverse to P_{Lac}. Thereafter, the mutagenized 1.5 kb DNA fragment was cloned into a pUC19 derivative containing the 0.5 kb non-coding promoter region of *petH* and P_{Lac} in tandem orientation. *Anabaena* 7119 mutant FNR proteins were prepared using as template the *petH* gene fragment ligated into the isopropyl-β-D-thiogalactoside (IPTG) inducible expression vector pTrc99A as above. Both series of mutations were verified by double-stranded dideoxy nucleotide sequencing. The yield was up to 20 mg PetH/liter of *E. coli*.

2.2. Enzyme purification

Recombinant PetF and FdxH from *Anabaena* 7120 were prepared as described [23]. For purification of recombinant *Anabaena* 29413 FNR, *E. coli* cells containing pFNRexp were washed in 10 mM Tris/1 mM EDTA buffer (TE), pH 7.5, and broken in a precooled French pressure cell. Recombinant FNR was obtained as 1:1 mixture of a 49 kDa- and a 35 kDa-form. Chromatography was performed with a Q-Sepharose fast flow column (Pharmacia) and FNR was eluted with 100 mM NaCl in TE-buffer pH 7.5. Yellow eluates, active in the diaphorase assay, were combined and concentrated with Centricon C3 microconcentrators (Amicon) to $\approx 150\ \mu M$ for all activity assays of the mutant proteins. For further purification, a ferredoxin affinity column was prepared with NHS-activated Sepharose (Pharmacia) coupled to PetF from *Anabaena* 7120. It was used to separate FNR from most of the *E. coli* proteins and to separate the 35 kDa protein from the 49 kDa FNR. The 35 kDa fraction was spectroscopically not distinguishable from the native flavoprotein (obtained from *Anabaena* 29413) with peaks at 393 and 458 nm; moreover, native and recombinant FNR yielded the same diaphorase and ferredoxin-dependent specific activities.

E. coli cells which overexpressed *Anabaena* PCC 7119 FNR were harvested from 4 l of IPTG induced cultures and resuspended in 50 mM Tris/HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM β-mercaptoethanol and 1 mM PMSF (buffer A). Cells were broken by sonication and centrifuged at $25\,000 \times g$ for 30 min at 4°C in order to separate the crude extract from the cell membranes. The supernatant containing FNR was collected on a DEAE 52 chromatography column and eluted applying a 0.02–0.2 M NaCl gradient. Two pools of FNR were obtained at different ionic strengths and corresponded to a 41 kDa and a 35 kDa FNR species. Fractions with the highest diaphorase activity (pool of 35 kDa FNR) were chromatographed on a Blue–Sepharose column and eluted with a gradient of 0.1–0.5 M NaCl in buffer A. Again the fractions that contained the highest FNR activity were collected and dialyzed against buffer A.

Polyacrylamide gel electrophoresis and UV–VIS spectra were used as criteria for purity of FNR. FNR concentration was estimated using an absorption coefficient (ε_{458}) of $9.7\ mM^{-1}\ cm^{-1}$.

2.3. Enzyme assays

Diaphorase activities of *Anabaena* 29413 FNR were determined with 2,6-dichlorophenol-indophenol (DCPIP) as artificial electron acceptor. The assay was performed in 50 mM Tris–HCl pH 8.0, 40 mM NaCl, 125 μM NADPH and 60 μM DCPIP. A slightly modified assay was used with the *Anabaena* 7119 mutant proteins (no salt addition, 100 μM NADPH and 95 μM DCPIP). The assay was started with 10 or 4 nM FNR, respectively, and followed spectroscopically at 620 nm.

The ferredoxin-dependent NADPH–cytochrome *c* reduction and $NADP^+$-photoreduction assays were performed essentially as described [24–26]. Recombinant *Anabaena* 7120 vegetative cell ferredoxin (PetF) and heterocyst ferredoxin (FdxH) were used in both assays. The modified reaction mixture for cytochrome *c* reduction contained 40 mM NaCl in addi-

tion to 8 μM PetF or 8 μM FdxH with the *Anabaena* 29413 PetH mutant proteins. In the case of *Anabaena* 7119 PetH mutants 40 μM PetF (without salt) were used. The reactions were started by addition of 4 nM FNR in both assays. The increase of absorbance was observed at 550 nm. For $NADP^+$-photoreduction, 200 nM of *Anabaena* 29413 FNR or *Anabaena* 7119 FNR and 2 or 5 μM PetF, respectively, were used instead, and NADPH formation was followed by the increase of absorbance at 340 nm.

3. Results

3.1. Heterologous expression and site-directed mutagenesis

The *petH* gene from *Anabaena* 29413 was cloned and sequenced in this study. The deduced amino acid sequence comprised 440 residues which were 98% identical to the *Anabaena* 7119 sequence and included the CpcD domain. The recombinant protein was also 137 amino acids longer than the presumably proteolytically processed, native product isolated from cyanobacteria [26]. The cloned *Anabaena* 7119 *petH* gene fragment started 126 bp upstream from the methionine codon encoding the first amino acid of the PetH protein [15], whereas in *Anabaena* 29413 the sequence preceding the first methionine was 0.5 kb as described in Section 2. The resulting recombinant *Anabaena* 7119 and *Anabaena* 29413 proteins were of different sizes: 41 and 49 kDa, respectively. Nevertheless, a similar FNR form of ≈ 35 kDa that could be a product of proteolytic cleavage was isolated from both *E. coli* cultures as well. All FNR forms have been isolated and they showed comparable enzymatic activities. We used the expression system to generate the following mutant proteins, all of which were implicated to participate at the ferredoxin binding site by chemical, cross-linking and X-ray model data. These were the positively charged residues R153, K209, K212, R214, K275, R401, K427, K430 and K431 which were supposed to interact with the two acidic surface domains around the iron–sulfur cluster of ferredoxin [13]. In *Anabaena* 29413 FNR, the above mentioned residues were replaced by neutral amino acid residues (Q and N), or an acidic residue (E) as in the case of *Anabaena* 7119 FNR.

3.2. Ferredoxin independent assays

As shown in Table 1, the diaphorase activities of the flavoprotein, which assay the electron transfer from reduced FNR to a small compound (DCPIP) remained more or less the same with the exception of mutation R214Q. Compared to the neutral replacements the reversed charge mutations (R153E; K212E) caused a larger decrease in the activity. Mutations in the patch involving residues in the 427–431 region did not produce any change in the diaphorase activity at all. The mutation at R214 deserves special attention. Although comparable amounts of R214Q-FNR were clearly present in crude extracts of *E. coli*, as determined by immunoblotting, spectroscopic analysis showed that the mutation interfered with integration of the FAD cofactor. A protein largely devoid of the FAD cofactor was purified with minimal enzymatic activity in the case of the neutral mutation (compare Table 1). All of the protein of the R214E mutation obtained was devoid of the cofactor and no activity could be measured with this mutant (data not shown). *Anabaena* 29413 FNR mutants were also assayed with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) as the electron acceptor in the diaphorase activity yielding quite similar, although not completely identical results (not shown).

3.3. Ferredoxin dependent assays

Light induced $NADP^+$-reduction by photosystem I is the main reaction catalyzed by FNR. Photoreduced ferredoxin acts as the electron donor of this reaction. The data in Table 2 obtained with both enzymes indicate that replacement of either one of the amino acid residues of FNR in the region of interaction with ferredoxin produced different rates of $NADP^+$ photoreduction. Those residues showing the largest changes in activity were R153, K212 and K430, which yielded only 21–30% of the wild type activity remaining upon replacement by a neutral residue and 5–15% upon the introduction of a negative charge. Other mutations, such as K209Q, caused intermediate effects on the activity. K275 showed wild type activity upon neutral substitution and 60% activity when a negative charge was introduced. The positive patch which includes residues 427–431 produced different

Table 1
Diaphorase activities of recombinant wild type and mutant ferredoxin:$NADP^+$reductase (PetH) of *Anabaena* 29413 and *Anabaena* 7119

PetH mutation		(spinach) [short FNR] amino acid number	DCIP diaphorase % activity	
			An 29413	*An* 7119
Wild type			100	100
R153Q		(33) [16]	86	
	R153E			67
K209Q		(88) [72]	98	
	K209E			88
K212Q		(91) [75]	88	
	K212E			65
R214Q		(93) [77]	18	
K275N		(153) [138]	103	
	K275E			92
	R401E	(275) [264]		77
	K427E	(301) [290]		100
K430Q		(304) [293]	99	
K431Q		(305) [294]	109	
	K431E			100

To allow comparisons, all values are given as percentage of recombinant *Anabaena* wild type PetH activity. Amino acid numbers refer to the deduced amino acid sequence of a full length clone of PetH (including the CpcD related domain). For comparison, the numbering of the corresponding residues of spinach FNR, and of the short form of *Anabaena* 7119 FNR used for crystallization [8] are given in parentheses and brackets, respectively. Reaction conditions are described in Section 2. The 100% value of the turnover number for *Anabaena* 29413 FNR was $42.0 \pm 1.8\,s^{-1}$ and for *Anabaena* 7119 FNR $73\,s^{-1}$. *An* = *Anabaena*.

Table 2
Electron transfer rates of recombinant wild type and mutant ferredoxin:$NADP^+$ reductase (PetH) of *Anabaena* 29413 and *Anabaena* 7119 in $NADP^+$ photoreduction and cytochrome *c* reduction

PetH mutation		(spinach) [short FNR] amino acid	$NADP^+$ photoreduction % activity (PetF)		Cytochrome *c* reduction % activity (PetF)		Cytochrome *c* reduction % activity (FdxH)
			An 29413	*An* 7119	*An* 29413	*An* 7119	*An* 29413
Wild type			100	100	100	100	100
R153Q		(33) [16]	21		7		7
	R153E			5		3	
K209Q		(88) [72]	66		13		10
	K209E			20		6	
K212Q		(91) [75]	30		6		5
	K212E			15		0	
K275N		(153) [138]	94		104		97
	K275E			60		10	
	R401E	(275) [264]		100		47	
	K427E	(301) [290]		90		25	
K430Q		(304) [293]	30		7		4
K431Q		(305) [294]	87		55		57
	K431E			105		20	

All values are given as percentage of the recombinant *Anabaena* wild type PetH activity. Amino acid residues numbering as in Table 1. The 100% value for $NADP^+$ photoreduction with *Anabaena* 29413 PetH in the presence of $2\,\mu M$ PetF was $35 \pm 1.5\,\mu mol$ NADPH formed $h^{-1}\,mg\,Chl^{-1}$. *Anabaena*-thylakoids ($10\,\mu g$ Chl) were washed with $NaNO_3$ to remove traces of bound ferredoxin and FNR. The 100% value for $NADP^+$ photoreduction by *Anabaena* 7119 thylakoids in the presence of $5\,\mu M$ ferredoxin was $109\,\mu mol$ NADPH formed $h^{-1}\,mg\,Chl^{-1}$. The 100% turnover number of cytochrome *c* reduction, as described in Section 2, was $161 \pm 7.6\,s^{-1}$ for *Anabaena* 29413 PetH and $147\,s^{-1}$ for *Anabaena* 7119 PetH. With FdxH and recombinant wild type PetH, the turnover number was $286 \pm 2.5\,s^{-1}$. *An* = *Anabaena*.

effects upon substitution. K427E showed wild type activity. No change was observed if K431 was replaced by both neutral and negatively charged residues. However, replacement of the neighboring K430 against Q exhibited a large decrease in activity.

To assay the reversed flow of electrons through FNR, which is of physiological importance as well, e.g. in non-photosynthetic tissues and cells, we used the cytochrome *c* reduction assay (Table 2). This assay showed qualitatively the same results, but the extent of inhibition was more pronounced. Even K431Q showed a significant decrease in the reaction rate (55% of wild type activity); the introduction of a negative charge in this position was even more severe. This pointed to some involvement of this lysine residue in ferredoxin binding. K275N again yielded wild type activities, but K275E showed a marked effect due to the charge reversal mutation. It should be emphasized, however, that data on the mutants were obtained with two enzymes (from *Anabaena* 29413 and 7119) and a structural characterization of the mutants is lacking.

Replacing the vegetative cell ferredoxin (PetF) by heterocyst ferredoxin (FdxH) yielded comparable results although the cytochrome *c* turnover number was increased with FdxH to 178% of PetF (Table 2).

4. Discussion

The involvement of electrostatic interactions between FNR and ferredoxin was proposed 25 years ago based on the stability of the complex in the presence of salt [2]. The formation of complexes of FNR with other electron transfer proteins, such as flavodoxin or rubredoxin suggested a certain degree of unspecificity in the recognition process.

From cross-linking and chemical modification experiments carried out with the spinach [27] and *Anabaena* [28,29] enzymes, several positively charged amino acid residues of FNR have been proposed to be involved in the interaction with ferredoxin. A model of how ferredoxin might dock onto spinach FNR which was consistent with published experimental results was presented by Karplus et al. [12,30]. More recently, based on the three dimensional structure of *Spirulina platensis* ferredoxin, Matsubara and coworkers proposed a model which essentially coincided with the previous ones for the FNR–ferredoxin complex [31].

Differential chemical modification has been used to map amino acid residues at the contact site between FNR and ferredoxin which form an electrostatically stabilized 1:1 complex. Based on X-ray structural models of spinach FNR and of ferredoxin from *Aphanothece sacrum*, De Pascalis et al. [13] showed that potential distribution and dipole moment orientation of ferredoxin suggested a complementary pattern at the binding site of FNR. As shown in Fig. 1(A), two regions of positive potential can be observed in FNR based on the local concentration of basic residues. Each one is located at either side of the redox active isoalloxazine ring of FAD: one at the right hand side of the model in Fig. 1(A) containing K427, K430 and K431 (*Anabaena* numbering, compare Table 1); a second patch of positive charges is located at the left hand side and contains K209, K212 and R214. Aliverti et al. [32] have demonstrated by site directed mutagenesis that K88 of spinach FNR (K209 of *Anabaena*) was important for the interaction with spinach ferredoxin, confirming previous cross-linking studies [33]. Other positively charged residues are distributed around the flavin ring such as R153, K275 and R401.

The X-ray model of ferredoxin from *Anabaena* (Fig. 1(B)) shows acidic residues grouped in patches located at certain distance from the iron sulfur center. These are residues D28, E31, E32, which are on the right hand side of Fig. 1(B). Another patch contains D67, D68 and D69 and is located on the opposite side of the iron sulfur center. A third patch includes the carboxy terminal residues of the protein with E94, one of the residues which was found to be absolutely essential for the electron transfer activity of this protein, and E95 which lacked an appreciable effect upon mutation [25,34].

Chemical modification and protection experiments showed that R77 and K294 (now R214 and K431) participated in ferredoxin binding to *Anabaena* FNR [28,29]. K53 of *Anabaena* (now K190 and not conserved in all FNR sequences), which was also modified by a pyridoxal phosphate derivative [28], was later found to be too far away from the suggested ferredoxin binding site in structural studies on crystallized FNR from *Anabaena* [8]. By mapping the ferredoxin binding site of spinach FNR, the lysine

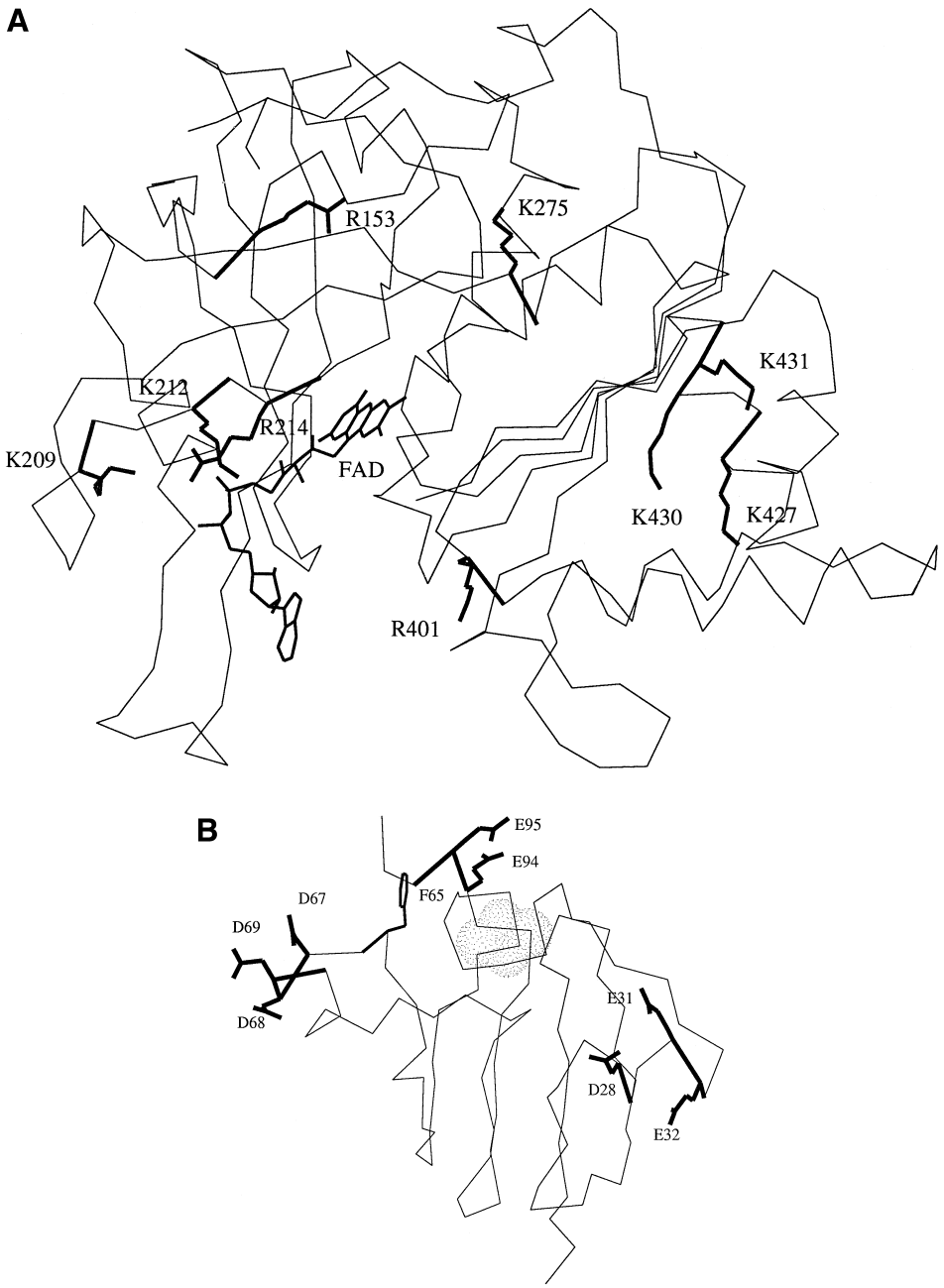

Fig. 1. Schematic view of the structure of *Anabaena* FNR and ferredoxin showing the alpha carbons and the side chains of residues which have been mutated in FNR (A) and those residues which are important for the electron transfer reaction or the interaction with FNR in ferredoxin (B).

residues 18, 33, 35 and 153 were protected in the complex with ferredoxin but not K304 and K305 possibly because of the absence of NADP(H) in the modification assay [13]. It has been shown that NADP(H) binding induces a conformational change of FNR [9].

The *petH* gene from both *Anabaena* strains was used for overexpression in *E. coli* and to generate

site-directed mutations. All of the above mentioned positively charged residues implicated in ferredoxin binding were substituted by neutral and acidic amino acid residues. Replacement of R214 (R93 in spinach) by glutamine yielded an FNR with a very low content of the FAD cofactor as shown by absorption spectroscopy, while replacement by glutamate produced an apoprotein completely devoid of the flavin group. It can be deduced from the X-ray model that the corresponding guanidino group of R214 stabilizes electrostatically the pyrophosphate group of the FAD cofactor [8], which seemed to be essential for interaction, similar to the corresponding R93 residue of the spinach enzyme [30].

Qualitatively $NADP^+$-photoreduction and the cytochrome *c* reduction rate were affected quite similarly by the mutations although the direction of electron transfer was reversed. However, the rate of cytochrome *c* reduction was inhibited more severely, especially by the exchanges at position R153, K209, K212, K275 and K430 to neutral or acidic amino acids. Neutral replacements are less likely to perturb the three dimensional structure or the stability of the proteins involved. The positively charged surface is essential for ferredoxin-dependent electron transfer by establishing electrostatic interactions with carboxyl groups at the surface around the iron–sulfur cluster of ferredoxin. To some extent K431 could also participate in ferredoxin binding or influence K430 in its neighborhood inhibiting electron flow (Table 2). The more pronounced effect observed on the activity of mutant K430 compared to K427 or K431 could be explained by the different orientation that these residues shown in the three-dimensional structure. K430 is pointing more towards the flavin ring and could occupy part of the putative ferredoxin-binding site (see Fig. 1(A)). The experiments do not rule out that electrostatic interactions of FNR with ferredoxin might be also necessary to facilitate electron transfer.

The heterocyst ferredoxin (FdxH), as shown previously, was almost twice as active in reversed electron flow than the vegetative cell ferredoxin (PetF). Despite the low degree of overall identity, the negatively charged residues at the surface around the iron–sulfur cluster are conserved in both FdxH and PetF.

Due to the irregular charge distribution the two molecules show a strong dipole moment which facilitates the mutual attraction at long distances. The dipoles orient the molecules in such a way that their charged surface patches are complementary. One possible orientation is that described in the model proposed by Karplus and Bruns [12] where K304 and K305 in spinach (K430 and K431 of *Anabaena*) interact with residues D28, D31 and D32 of *Aphanothece* ferredoxin. Residues K85, K88, K91 and R93 of spinach FNR (K206, K209, K212 and R214 of *Anabaena*) would interact with the carboxy terminal glutamate residues (E94, E95) in ferredoxin.

Electron transfer might require tighter interaction between the two proteins than just electrostatic interactions so that the two cofactors get closer together. This probably occurs through the release of water molecules from the protein–protein interface [35] which might establish hydrophobic interactions between non polar amino acids in the region around the flavin and iron sulfur center. It should be mentioned in this respect that the aromatic side chain of F65 of *Anabaena* ferredoxin (PetF) was found to be essential for electron transfer to FNR [25,36]. Moreover, at ionic strength above 100 mM, the complex was more stable than expected based only on electrostatic interactions. This has been taken as an indication that hydrophobic interactions contribute significantly to the complex stability and, probably, to the electron transfer reaction [37].

The ionic strength effect observed in many complexes between wild type FNR and mutant ferredoxins would be due to the need of the two proteins to dissociate if they form an unproductive complex and also to rearrange in a less stable complex based on electrostatic interactions [34,36,37]. FNR residues involved in hydrophobic interactions with ferredoxin remain to be elucidated by site directed mutagenesis.

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