ELSEVIER

Biochimica et Biophysica Acta 1231 (1995) 335–341

BBA Biochimica et Biophysica Acta

Amino acid residues involved in functional interaction of vegetative cell ferredoxin from the cyanobacterium *Anabaena* sp. PCC 7120 with ferredoxin:NADP reductase, nitrite reductase and nitrate reductase ☆

Stefan Schmitz, Herbert Böhme *

Botanisches Institut der Universität Bonn, Kirschallee 1, D-53115 Bonn, Germany

Received 11 April 1995; accepted 15 June 1995

Abstract

During evolution, cyanobacterial [2Fe-2S]-ferredoxins have developed structural features that enable them to form 1:1 complexes with different redox partners, which are mainly stabilized by electrostatic interactions. Three important ferredoxin-binding proteins are ferredoxin:NADP reductase (FNR), nitrite reductase (NIR) and nitrate reductase (NAR). Using site-directed mutagenesis with recombinant ferredoxin from vegetative cells (PetF) of *Anabaena* 7120, we measured the in vitro activities of these enzymes in ferredoxin-dependent steady-state assays. Aside from a major contribution of surface charge, electron transfer from reduced ferredoxin to all three redox partners was severely inhibited by amino acid substitutions at positions E94 and F65. However, mutations at these and other sites affected rates of electron transport to FNR, NIR and NAR differently. This suggested a common, but not identical, ferredoxin binding-domain on FNR, NIR and NAR. Residues E94 and F65 were previously shown to be required for rapid electron transfer to FNR [Hurley et al. (1993) Biochemistry 32, 9346–9354]. We show here additionally that the reversed electron flow from NADPH-reduced FNR to oxidized ferredoxin was strongly stimulated by the mutated proteins E94Q and F65A that showed almost no electron transport capacity to oxidized FNR. This supports a conformational change of the FNR induced by NADPH-binding and subsequent electron transfer supporting dissociation of the FNR-ferredoxin complex.

Keywords: Ferredoxin; Ferredoxin:NADP reductase; Nitrate reductase; Nitrite reductase; Common binding site; (*Anabaena*)

1. Introduction

In cyanobacteria and higher plants, [2Fe-2S]-ferredoxins serve as electron donors to a number of redox proteins. Among these are ferredoxin:NADP reductase (FNR), nitrite reductase (NIR), sulfite reductase, glutamate synthase, thioredoxin reductase and in cyanobacteria also nitrate reductase (NAR) [1]. Cyanobacterial 'plant-type' ferredoxins form a group of highly conserved soluble electron carriers, characterized by their low molecular weight and redox potential. All known high-resolution structures exhibit the same global folding pattern, although vegetative cell ferredoxin (PetF) and heterocyst ferredoxin (FdxH) from *Anabaena* 7120 [2,3] showed an identity of only 51% on the amino-acid level [4]. Both ferredoxins were designed for different metabolic pathways in vegetative cells and heterocysts of filamentous cyanobacteria, respectively [5–7]. One common feature of the ferredoxins is their high negative net charge due to conserved acidic residues. Hence, it is not surprising that all ferredoxin-binding studies revealed the contribution of electrostatic interactions [8]. Along with other results, this led to the proposal of a common ferredoxin-binding site on different ferredoxin-interacting enzymes [9]. Research aimed to reveal specific sites for interaction of ferredoxin and ferredoxin-binding proteins has concentrated mostly on FNR, where the tertiary structure of the spinach enzyme is known [10]. Using laser flash photolysis to reduce ferredoxin, electron transport of PetF-mutants of *Anabaena* 7120 to FNR of *Anabaena* 7119 clearly showed that a negative charge at position 94 as well as an aromatic residue at position 65 of PetF were crucial to rapid electron transfer to FNR [11–13]. Both residues were in the direct vicinity of the [2Fe-2S] cluster and thought to be a

☆ This paper is dedicated to Prof. Dr. Dr. h.c.mult. A. Trebst on the occasion of his 65th birthday

* Corresponding author. Fax: +49 228 735513.

SSDI 0005-2728(95)00097-6

part of the binding domain of PetF. However, De Pascalis et al. [14] found selective protection of residues E29, E30, D34 and D65 in spinach ferredoxin (corresponding to residues 31, 32, 36 and 67 in *Anabaena* PetF) when bound to spinach FNR, but not of the C-terminal glutamates, such as E94. Based on these results, a binding model was proposed in which the complementary surface potential of ferredoxin and FNR directed the docking process.

Other ferredoxin-binding proteins are less well studied. Photosystem I of *Synechocystis* sp. PCC 6803 was shown to be influenced in its ferredoxin-binding capability mainly by the ionic strength of the medium [15]. Residue E93 of *Synechocystis* ferredoxin (corresponding to E95 of *Anabaena* 7120 PetF) was identified by chemical cross-linking to bind to K106 of the PsaD-subunit of the *Synechocystis* reaction center [16].

Cyanobacterial nitrite reductase (NIR) is a monomeric 52–54 kDa protein with a siroheme and a [4Fe-4S] cluster as prosthetic groups [17,18]. Spinach NIR competed with FNR in cross-linking to ferredoxin [19]. Antibodies raised against spinach FNR also recognized spinach NIR and glutamate synthase (GOGAT) [20]. Carboxyl groups on the ferredoxin were necessary for binding to NIR [9] and recently the ionic strength dependence of electron transport from ferredoxin to the siroheme group of NIR has been demonstrated [21].

Nitrate reductase (NAR) of higher plants is a NAD(P)H-dependent protein. In cyanobacteria it was shown to be ferredoxin-linked [22]. NAR of *Plectonema boryanum* has a molecular mass of 85 kDa and a molybdenum cofactor in addition to two [2Fe-2S] clusters [23]. To our knowledge, specific binding properties have never been investigated.

Spinach ferredoxin-thioredoxin reductase has also been studied by selective amino acid protection [24]. A site including residues E94-E96 was protected, which was different from the results obtained with FNR. De Pascalis et al. [24] proposed an overlapping binding site including the [2Fe-2S] cluster and emphasized the possibility of different productive complexes for a given protein pair.

Because of the general importance of electrostatic interactions for ferredoxin binding, we decided to exchange conserved acidic residues on *Anabaena* PetF with the corresponding amides and determine the effects on electron transfer to FNR, NIR and NAR. All exchanged acidic residues belong to two domains of negatively charged surface potential surrounding the [2Fe-2S] cluster [14].

2. Materials and methods

2.1. Culture conditions

Anabaena variabilis (ATCC 29413) was continously grown in glass tubes containing 200 ml Arnon medium [25] without combined nitrogen. The cultures were bubbled with air enriched with 1% (v/v) CO_2 and illuminated with white fluorescent light (100 μE m^{-2} s^{-1}) at 30°C. One of these cultures was used to inoculate 10 liter of BG1b medium supplied with 18 mM $NaNO_3$ [25]. Growth conditions were the same as for 200 ml cultures. After 24 h incubation at 35°C the NIR and NAR activities were fully induced. The culture was harvested by continuous flow centrifugation.

2.2. Site directed mutagenesis

Oligonucleotide-directed mutagenesis was performed using the PCR variant of the unique site elimination method [26]. For selection, we used a primer that changes the unique *Nde*I site of pUC18/19 to *Dra*III. Oligonucleotide primers for mutagenesis were obtained from Pharmacia (Freiburg) or MWG (München). For all mutagenesis experiments the plasmid pAn 665.1 was used containing the complete *petF* gene cloned into pUC19 [27]. DNA sequence analysis (^{T7}sequencing kit, Pharmacia) verified successful mutagenesis. In vitro mutated plasmids were propagated in *E. coli* strain BMH 71–18 mutS (Boehringer Mannheim). Other molecular biology procedures followed standard protocols [28]. Overexpression of recombinant ferredoxin was performed after transformation of *E. coli* strain MC 1061 [29,30]. With freshly transformed cells 500 ml of 2 × YT medium in the presence of ampicillin (100 μg/ml) were inoculated and grown for 18 h at 37°C with continous shaking (150 rpm). The yield was up to 50 mg holo-ferredoxin per liter of medium. Harvesting, cell disruption and purification of recombinant ferredoxin were performed as described [30]. After two Q-sepharose columns (1.5 × 6.5 cm, Pharmacia LKB) the ferredoxin preparation (≈ 400 μM) was more than 90% pure as judged from native polyacrylamide gel electrophoresis [27].

2.3. Isolation of enzymes and assays

Ferredoxin:NADP reductase: NADP photoreduction was determined with washed thylakoids (12.6 μg Chl *a*/ml) obtained during purification of nitrate reductase (see below). Conditions are described in Schmitz et al. [27]; however, both recombinant FNR and ferredoxin were used at concentrations of 2 μM. Cytochrome *c* reduction was determined at 550 nm [31]. 8 μM ferredoxin and 10.5 nM recombinant FNR were added to the reaction mixture. Isolation and purification of recombinant FNR from *A. variabilis* will be published elsewhere. Cytochrome *c* reduction and NADP photoreduction assays with FNR purified from *A. variabilis* yielded the same results as with the recombinant protein.

Nitrite reductase: 21 g of *A. variabilis* cells were disrupted by French press treatment. Thylakoids were spun down by ultracentrifugation (126000 × *g*, 75 min). A 21-fold purification of NIR was obtained after complete re-

moval of cyanobacterial ferredoxins by two subsequent Q-Sepharose anion exchange columns (elution of NIR with 300 mM NaCl in 10 mM Tris-HCl/1mM EDTA (TE) buffer (pH 7.5)). Measurements were performed in 8 ml vials, closed with septum stoppers. The reaction mixture (1 ml) contained: 10 mM Tris-HCl (pH 8.0), 0.2 mM KNO_2, recombinant ferredoxin at different concentrations (0.2–50 μM) and partially purified NIR (12 μg protein, methyl viologen dependent activity = 10 mU). The reaction mixture was made anaerobic with argon and the reaction was started by addition of 5 mM $Na_2S_2O_4$ (dissolved in argon-flushed water). Aliquots of 300 μl were taken after 1 and 6 min of reaction time at 30°C under continuous shaking (10 rpm). The reaction was immediately stopped by vigorous mixing in air. Proteins were precipitated by addition of 50 μl of 1 M $ZnSO_4$, 50 μl of 1 M NaOH and subsequent centrifugation. 250 μl of the clear supernatant was used for nitrite determination [32]. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the reduction of 1 μmol nitrite per min at 30°C.

Nitrate reductase: Partially purified NAR was prepared basically according to Martin-Nieto et al. [33]. 16 g of BG 11-grown *Anabaena variabilis* cells were suspended in TE buffer (pH 7.5) + 1 mM PMSF and disrupted in a French press. After 80 min of centrifugation at 40000 × g, about 90% of NAR activity was found to be thylakoid-bound. Treatment of the thylakoid membranes with 1 M $NaNO_3$ released about 80% of NAR activity in the supernatant after ultracentrifugation (100000 × g, 90 min). $NaNO_3$-washed thylakoids were also used for NADP-photoreduction measurements after suspending them in 30 mM Tricine-KOH/10 mM $MgCl_2$/5 mM Na_2HPO_4 (pH 8.0). Differential ethanol precipitation (50–75%) and Q-Sepharose anion-exchange chromatography removed all cyanobacterial ferredoxin. NAR was eluted at 200 mM NaCl. A 500-fold purified NAR (≈ 50% yield) was obtained and used for the assays. Because dithionite was known to impair ferredoxin-dependent NAR activity [34] ferredoxin was reduced via recombinant *Anabaena* FNR and an NADPH-regenerating system with glucose-6-phosphate dehydrogenase under anaerobic conditions. The assay contained in a total volume of 0.5 ml: 10 mM Tris-HCl (pH 8.0), 4 mM glucose 6-phosphate, 0.5 mM NADP, 10 mM $NaNO_3$, 0.26 μM FNR, 2 μg glucose 6-phosphate dehydrogenase (Boehringer Mannheim) and 15 μM ferredoxin. For determination of K_M and v_{max}, ferredoxin concentration was varied between 1 and 100 μM. After sparging with argon for 2 min, the reaction was started by addition of partially purified NAR (6.2 μg protein) and carried out for 3 min at 30°C with continous shaking (10 rpm). To stop the reaction, 300 μl were taken off, added to 50 μl of 1 M NaOH and mixed vigorously. After addition of 50 μl of 1 M $ZnSO_4$ and subsequent centrifugation, proteins were precipitated. The generated nitrite was determined in 250 μl of the clear supernatant [32]. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the reduction of 1 μmol nitrate per min at 30°C.

All results presented were the average of 3–6 independent measurements.

3. Results and discussion

Since [2Fe-2S]-ferredoxins are highly negatively charged proteins, it is reasonable to assume that electrostatic interactions are a decisive element in their ability to bind to different redox proteins. Recent observations with Photosystem I of *Synechocystis* PCC 6803 [15,35], FNR of *Anabaena* PCC 7119 [11] and spinach NIR [21] clearly underline this hypothesis. In Fig. 1 we show that electron transport from ferredoxin to NIR and NAR of *A. variabilis* was strongly dependent on the ionic strength of the medium in which the reaction was carried out. At very low (below approx. 40 mM NaCl) and high ionic strength the electron transfer rate decreased in both the NIR and NAR assays (compare Fig. 8 in Ref. [21]). Measurements of the electron transfer kinetics of ferredoxin to FNR yielded similar results [11]. The data suggest that a high ionic strength of the medium weakens or even prevents binding of ferredoxin to its reaction partners (FNR, NIR and NAR), while an ionic strength around zero makes it more difficult for the protein-protein complex to dissociate after electron transfer. Both conditions slow down the rate constant.

Because charged residues contribute to the binding of ferredoxin to its physiological redox partners, we decided to exchange acidic residues absolutely conserved among 20 cyanobacterial ferredoxin sequences to uncharged amides in order to examine their specific role in different electron transport systems. To exclude major misfoldings,

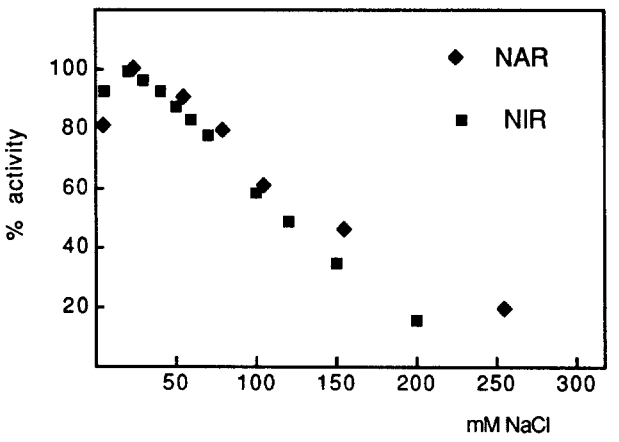

Fig. 1. Effect of ionic strength on electron transfer from recombinant ferredoxin (PetF) to nitrate reductase (NAR, ◆) and nitrite reductase (NIR, ■), respectively. PetF concentration was 7 μM in the NAR and 5 μM in the NIR assay. Ionic strength was adjusted by addition of aliquots of 5 M NaCl to the reaction mixture. The 100% value for NAR activity was 18.4 U/mg and for NIR activity 0.7 U/mg.

Table 1
Electron transfer rates of recombinant wild type and mutated ferredoxins in NADP-photoreduction, cytochrome *c* reduction, nitrite- and nitrate-reductase assays

	Ferredoxin:NADP reductase		Nitrite reductase	Nitrate reductase
	NADP photoreduction	cytochrome *c* reduction		
Without PetF	< 5	< 5	0	0
PetF	100 ± 5	100 ± 8	100 ± 4	100 ± 6
E10K/A11K	90 ± 3	114 ± 6	102 ± 5	72 ± 7
D22N/D23N	81 ± 1	119 ± 9	89 ± 6	97 ± 4
D28N	78 ± 9	145 ± 8	84 ± 8	131 ± 9
E31Q/E32Q	69 ± 4	142 ± 12	83 ± 5	99 ± 8
D67N/D68N	61 ± 6	43 ± 5	94 ± 3	n.m.
D67N	65 ± 9	64 ± 4	n.d.	n.m.
E94Q/E95Q	< 5	216 ± 17	17 ± 3	18 ± 1
E94Q	< 5	196 ± 3	19 ± 1	46 ± 2
F65A	< 5	167 ± 22	32 ± 2	36 ± 1
F65I	< 5	142 ± 12	55 ± 4	65 ± 5
F65W	108 ± 7	83 ± 12	96 ± 6	48 ± 7
F65Y	93 ± 5	94 ± 4	97 ± 9	78 ± 1
FdxH	54 ± 4	156 ± 11	20 ± 1	24 ± 1

For reasons of comparison, all values were measured in each assay at a constant ferredoxin concentration and given as percentage related to wt PetF activity = 100%. The 100% values were for NADP photoreduction: 13.3 μmol NADPH formed/h per mg Chl *a*; cytochrome *c* turnover number: 46.5/s; nitrite reductase activity: 0.5 U/mg protein; nitrate reductase activity: 2.6 U/mg protein. NADP-photoreduction was measured using thylakoids washed with $NaNO_3$ (see Materials and Methods). The ferredoxin concentrations in the four assays were in μM: 2, 8, 2.5 and 15, respectively. Reaction conditions were as described in Materials and methods.
n.d. = not determined.
n.m. = not measureable.

UV/VIS spectra of all mutated ferredoxins were recorded, but in no case were differences from the wild-type spectrum detected (data not shown). Hurley et al. [11] showed for the mutant ferredoxins E94K and F65A that reduction potentials remained unaltered. Also, long-term storage at −20°C showed no higher instability for any of the mutated proteins which would be recognized by eventual loss of the [2Fe-2S] center.

3.1. Electron transfer to FNR

Using a different experimental system, Hurley et al. [11–13] published results strikingly similar to our findings. Both a negative charge at residue 94 (a conserved glutamate in all ferredoxins) and an aromatic residue at position 65 (a conserved phenylalanine) were necessary for efficient electron transfer from reduced *Anabaena* 7120 ferredoxin to FNR from *Anabaena* 7119. Our experiments with ferredoxin reduced by Photosystem I confirmed these observations (Table 1, column 1). PetF mutants E94Q, F65A and F65I showed no detectable activities in NADP photoreduction. Replacing F65 with either W or Y restored wild-type (wt) rates. All other mutants with singly or doubly removed negative charges also had lower activities than wt PetF but certainly not to that extent. These exchanges alter the dipole moment of PetF, which may be a critical value for the very first steps in the ferredoxin-FNR docking process [14].

Surprisingly, the second FNR-dependent assay, the cytochrome *c* reduction, yielded completely different results (Table 1, column 2). It showed activities more or less inverse to NADP photoreduction. Compared to wt PetF, nearly all mutants exhibited higher turnover numbers: e.g., mutant E94Q/E95Q was the most active ferredoxin in cytochrome *c* reduction –the one that was completely inactive in NADP photoreduction. Diaphorase activity of FNR, using a redox dye instead of cytochrome *c* as electron acceptor, performed with each of the modified ferredoxins gave qualitatively the same results (data not shown, cf. Ref. [27]). This shows that cytochrome *c* reduction is a specific assay for the interaction of ferredoxin with FNR and detects 'reversed' electron transport from FNR (reduced by NADPH) to oxidized ferredoxin. Mutants that were inactive in NADP photoreduction (like E94Q and F65A) stimulated electron flow to cytochrome *c* because *only* the reversed electron transport worked, while the back reaction was largely impaired. This means that for binding and electron transfer from FNR_{red} to oxidized ferredoxin (as measured in cytochrome *c* reduction) different amino acid residues are essential than for electron transfer of reduced ferredoxin to FNR_{ox} (measured in NADP photoreduction and laser flash photolysis). Probably, binding of NADPH and subsequent electron transport induces a conformational change within the FNR-ferredoxin complex [36], facilitating the release of reduced ferredoxin.

As shown before for the spinach enzymes, reduced ferredoxin binds well to the binary FNR-$NADP^+$ complex [37,38]. Upon formation of the ternary complex, a change in redox potentials increases the thermodynamic driving force for electron transfer from reduced ferredoxin to FNR/$NADP^+$. After electron transfer, dissociation of the oxidized ferredoxin (a rate-limiting step) is favored by the negative cooperativity between ferredoxin-and NADP(H)-binding sites on FNR (cf. citations in Ref. [9]).

According to that, the different reactivities of PetF and FdxH with FNR are a matter of interest. PetF was twice as active in NADP-photoreduction while FdxH exhibited a considerably higher turnover number in cytochrome *c* reduction (Tab. 1). PetF seems to be preferentially designed for the 'common' direction of electron flow as in NADP photoreduction. On the contrary, FdxH is more adapted to reverse electron transport. This may be physiologically important, because in heterocysts, the compartment where FdxH is synthesized, NADPH is produced by carbon degradation via a highly active oxidative pentose-phosphate pathway [39]. Electrons are transferred to FdxH via FNR, which is approximately 10-times more abundant in heterocysts as shown on the level of protein content and enzymatic activity [40]. When reduced, FdxH functions as

efficient and immediate electron donor to nitrogenase [5]. Hence, FdxH was designed not only to deliver electrons to nitrogenase but also to receive electrons from NADPH via FNR. We showed previously that lysine residues 10 and 11 of FdxH were crucial to binding and electron transfer to nitrogenase reductase [27]. When we exchanged the corresponding amino acids in PetF at these positions –EA to KK –none of the reactions with FNR, NIR and NAR was influenced to any significant extent (Table 1). This emphasizes the importance of lysines 10 and 11 in docking FdxH exclusively to nitrogenase reductase.

PetF mutant D67N was the only one that showed reduced activity in both FNR-dependent assays (Table 1). D67 is in the vicinity of the Fe-S cluster just as residues E94 and F65. However, the change of the adjacent residues D68/D69 to KK resulted only in little perturbation of the PetF/FNR interaction; at higher ionic strength this mutant was less active than wt ferredoxin [11]. Our data suggest that D67 contributes significantly to FNR binding in the reduced as well as in the oxidized state of PetF. Interestingly, residue D65 in spinach ferredoxin (= D67 of *Anabaena* PetF) was the most protected residue in differential chemical modification of acidic residues when bound to spinach FNR [14]. Although this was a non-functional complex, as it consisted of oxidized FNR and oxidized ferredoxin in the absence of NADP(H) –which was criticized by Aliverti et al. [41] –our results also suggest participation of D67 when a complex with FNR is formed.

3.2. Electron transfer to nitrite reductase

In order to test the ability of the ferredoxin mutant proteins to transfer electrons to NIR they were reduced with sodium dithionite. The K_M for PetF electron transfer to NIR was determined as 2.5 μM, which is in good agreement with results for the *Anabaena* PCC 7119 enzyme [42]. Rate constants for a ferredoxin concentration of 2.5 μM are presented in Table 1, column 3; the kinetic parameters (K_M and v_{max}) are given in Table 2A. Comparison of NADP photoreduction and the NIR assay showed striking similarities, although the two assay systems are completely different. For the mutants E10K/A11K, D22N/D23N, D28N, E31Q/E32Q, D67N/D68N, F65W and F65Y, K_M and v_{max} stayed within the wt PetF range. The slight decrease in NIR activity for D22N/D23N, D28N and E31Q/E32Q can again be explained by the altered surface charge of PetF as in the interaction with FNR. Significant activity changes only occurred for mutants E94Q/E95Q, E94Q, F65A and F65I. For efficient ET to NIR, again both a negative charge at position 94 and an aromatic residue at position 65 on PetF were necessary – identical to the results obtained with FNR. This supports the hypothesis that the ferredoxin-binding sites at FNR and NIR, two non-homologous redox proteins, are structurally related, but not completely identical. One difference concerned residue D67. Even the double mutant D67N/D68N showed wild-type behavior (Tables 1 and 2A). This can be interpreted in two ways: (i) these residues are not involved in interaction with NIR; (ii) they belong to the domain that binds to NIR but the negative charge is not important. Only additional mutations may answer this question. Another difference between NADP photoreduction and the NIR assay concerned the extent of inhibition. While electron transport to FNR was strongly inhibited in mutants E94Q, F65A and F65I, these amino acid exchanges still allowed some electron flow to NIR. Especially the kinetic parameters for F65A and F65I (Table 2A) showed only a slight increase in the K_M values (4.7 μM and 3.7 μM, respectively). This suggests that binding of PetF to NIR is not hindered to a large extent by these mutations, but a conformation between the two proteins optimal for electron transport cannot be achieved. Chemical modification of spinach NIR with *N*-bromosuccinimide revealed a tryptophan residue crucial to ferredoxin binding [43]. Despite possible differences between the cyanobacterial and the higher plant enzyme, it is possible that this residue of NIR is directly interacting with F65 of the spinach ferredoxin.

On the other hand, FdxH was less active in this assay than in NADP photoreduction when compared to PetF. Especially its K_M value of 16.5 μM (Table 2A) was much higher than for PetF. This is not very surprising, since FdxH as a heterocyst protein could never interact with NIR, which is expressed only in vegetative cells. However, the 'natural variant' FdxH clearly showed that a negative charge at position 94 and an aromatic residue at position 65 of a ferredoxin were not sufficient for effective electron transport to NIR. FdxH, which contains both F65 and E94, fulfils this 'minimal assumption' and its overall

Table 2
Kinetic parameters for the nitrite-reductase and nitrate-reductase assays

A Nitrite-reductase

Ferredoxin	K_M (μM)	v_{max} (U/mg)	Ferredoxin	K_M (μM)	v_{max} (U/mg)
PetF	2.5	1.00			
E10K/A11K	2.5	1.02	E94Q	8.5	0.41
D22N/D23N	3.0	1.00	F65A	4.7	0.46
D28N	3.1	0.94	F65I	3.7	0.68
E31Q/E32Q	3.7	1.03	F65W	2.8	1.02
D67N/D68N	2.9	1.02	F65Y	2.5	0.97
E94Q/E95Q	8.1	0.35	FdxH	16.5	0.75

B Nitrate-reductase

Ferredoxin	K_M (μM)	v_{max} (U/mg)
PetF	15.5	6.50
E94Q/E95Q	14.8	1.45
E94Q	8.4	2.08
FdxH	13.9	1.37

Apparent K_M and v_{max} for wild-type and mutant ferredoxins were calculated using Woolf plots ([S]/v against [S]).

structure is also very similar to that of PetF. Obviously, other residues specific for PetF must participate in the interaction between ferredoxin and NIR. Such PetF-specific residues remain to be elucidated, since all mutations except E10K/A11K were performed at positions conserved in PetF-type as well as in FdxH-type ferredoxins.

3.3. Electron transfer to nitrate reductase

For measurements of electron transport to NAR we reduced ferredoxin via recombinant FNR from *Anabaena* 29413 by a NADPH-regenerating system. This gave rise to the possibility that interactions of NAR as well as of FNR with ferredoxin might determine the rate constant. The assay conditions were therefore chosen to be rate-limiting for the ferredoxin/NAR electron transfer and FNR was provided in excess. However, ferredoxin mutants D67N and D67N/D68N showed no saturation kinetics with increasing amounts of added FNR, because these amino acid exchanges caused a reduced capacity to accept electrons from FNR (see Table 1, column 2). So, these two mutants could not be directly compared to the others. However, an activity of about 80% of wt PetF measured under these conditions allowed the conclusion that D67 played no key role in the ferredoxin-NAR interaction.

As presented in Table 1 (column 4), the PetF double mutant E94Q/E95Q strongly inhibited electron transfer to NAR (reduction to 18% of wtPetF). In contrast to NADP photoreduction and the NIR assay, the corresponding single mutant E94Q did not show the same extent of inhibition (64% of wt PetF). In this case, residue E95 seemed to contribute additionally to the ferredoxin-NAR interaction. The F65A exchange resulted in a reduction of the reaction rate to 36%. F65I still allowed 65% of wt activity. In contrast to FNR and NIR, not only the aromaticity of a residue at this position was important but also the size of the amino acid: F65W yielded only 48%, even F65Y reduced activity to 78%. For optimal electron transfer from ferredoxin to nitrate reductase, a phenylalanine at position 65 was needed; replacement with other aromatic residues did not restore wt PetF properties, in contrast to the situation with FNR or NIR. This could be one explanation as to why F65 is absolutely conserved among all known cyanobacterial plant-type ferredoxins. Only two higher plant ferredoxins have a tyrosine at this position, but higher plant NAR is not ferredoxin-dependent. In general, NAR exhibited a ferredoxin-binding site similar to FNR and NIR. Such results were also obtained for cytochrome *c*-binding proteins, where binding sites were not identical but overlapped [44].

As observed with the NIR assay, FdxH was much less active than PetF in electron transport to NAR (Table 1). The kinetic parameters (Table 2B) show that the reasons for this similar behavior were quite different. The K_M of PetF for electron transfer to NAR was 15.6 μM, while FdxH exhibited 13.9 μM. Mutant E94Q, which acted as an inhibitor, too, had a K_M of only 8.4 μM. In all cases, v_{max} was decreased significantly. A good adaptation of a protein to its substrate is usually indicated by a small K_M. The relatively high K_M of the PetF/NAR couple and the low K_M of PetF/NIR may have physiological significance by keeping the intracellular nitrite concentration at a low level. Nitrite is a potential mutagenic agent. In the metabolic pathway of assimilatory nitrate reduction via NAR and NIR, the different K_M values of the two enzymes for ferredoxin may help to provide NIR preferentially with electrons if its second substrate (nitrite) is present in micromolar amounts. A higher efficiency of electron flow from reduced ferredoxin to NAR –as shown for mutant D28N –might be not advantageous for the cyanobacterium where ferredoxin must serve many and varied roles in the distribution of high-energy electrons.

Acknowledgements

We are especially grateful to Dr. J.K. Hurley and Prof. G. Tollin (at the University of Arizona) and Prof. J.L. Markley (University of Wisconsin) for making available to us samples of the ferredoxins F65A,I,W,Y. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Bo 660), the Graduiertenkolleg in Bonn and the Fond der Chemischen Industrie. Parts of this work were presented at the VIII ISPP in Urbino (1994), Italy.

References

[1] Rogers, L.J. (1987) in the Cyanobacteria (Fay, P. and Van Baalen C., eds.), pp. 35–67, Elsevier, Amsterdam.
[2] Rypniewski, W.R., Breiter, D.R., Benning, M.M., Wesenberg, G., Oh, B.-H., Markley, J.L., Rayment, I. and Holden, H.M. (1991) Biochemistry 30, 4126–4131.
[3] Jacobson, B.L., Chae, Y.K., Markley, J.L., Rayment, I. and Holden, H.M. (1993) Biochemistry 32, 6788–6793.
[4] Böhme, H. and Haselkorn, R. (1988) Mol. Gen. Genet. 214, 278–285.
[5] Schrautemeier, B. and Böhme, H. (1985) FEBS Lett. 184, 304–308.
[6] Böhme, H. and Schrautemeier, B. (1987) Biochim. Biophys. Acta 891, 1–7.
[7] Böhme, H. and Schrautemeier, B. (1987) Biochim. Biophys. Acta 891, 115–120.
[8] Knaff, D.B. and Hirasawa, M. (1991) Biochim. Biophys. Acta 1056, 93–125.
[9] Hirasawa, M., Boyer, I.M., Gray, K.A., Davis, D.J. and Knaff, D.B. (1986) Biochim. Biophys. Acta 851, 23–28.
[10] Karplus, P.A., Daniels, M.J. and Herriot, J.R. (1991) Science 251, 60–66.
[11] Hurley, J.K., Salomon, Z., Meyer, T.E., Fitch, J.C., Cusanovich, M.A., Markley, J.L., Cheng, H., Xia, B., Chae, Y.K., Medina, M., Gomez-Moreno, C. and Tollin, G. (1993) Biochemistry 32, 9346–9354.
[12] Hurley, J.K., Cheng, H., Xia, B., Markley, J.L., Medina, M., Gomez-Moreno, C. and Tollin, G. (1993) J. Am. Chem. Soc. 115, 11698–11701.
[13] Hurley, J.K., Medina, M., Gomez-Moreno, C. and Tollin, G. (1994) Arch. Biochem. Biophys. 312, 480–486.

[14] De Pascalis, A.R., Jelesarov, I., Ackermann, F., Koppenol, W.H., Hirasawa, M., Knaff, D.B. and Bosshard, H.R. (1993) Protein Sci. 2, 1126–1135.
[15] Sétif, P.Q. and Bottin, H. (1994) Biochemistry 33, 8495–8504.
[16] Lelong, C., Sétif, P.Q., Lagoutte, B. and Bottin, H. (1994) J. Biol. Chem. 269, 10034–10039.
[17] Méndez, J.M. and Vega, J.M. (1981) Physiol. Plant. 52, 7–14.
[18] Arizmendi, J.M. and Serra J.L. (1990) Biochim. Biophys Acta 1040, 237–244.
[19] Privalle, L.S., Privalle, C.T., Leonardy, N.J. and Kamin, H. (1985) J. Biol. Chem. 260, 14344–14350.
[20] Hirasawa, M., Gray, K.A., Sung, J.D. and Knaff, D.B. (1989) Arch. Biochem. Biophys. 275, 1–10.
[21] Hirasawa, M., Tollin, G., Salamon, Z. and Knaff, D.B. (1994) Biochim. Biophys. Acta 1185, 336–345.
[22] Hattori, A. and Myers, J. (1967) Plant Cell Physiol. 8, 327–337.
[23] Mikami, M. and Ida, S. (1984) Biochim. Biophys. Acta 791, 294–304.
[24] De Pascalis, A.R., Schürmann, P. and Bosshard, H.R. (1994) FEBS Lett. 337, 217–220.
[25] Castenholz, R.W. (1988) Methods Enzymol. 167, 68–93.
[26] Deng, W.P. and Nickoloff, J.A. (1992) Anal. Biochem. 200, 81–88.
[27] Schmitz, S., Schrautemeier, B. and Böhme, H. (1993) Mol. Gen. Genet. 240, 455–460.
[28] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[29] Casadaban, M.J. and Cohen, S.N. (1980) J. Mol. Biol. 138, 179–207.
[30] Böhme, H. and Haselkorn, R. (1989) Plant. Mol. Biol. 12, 667–672.
[31] Shin, M. (1971) Methods Enzymol. 23, 440–447.
[32] Snell, F.D. and Snell, C.T. (1949) Colorimetric Methods of Analysis, Vol. 3, pp. 804–805, Van Nostrand, New York.
[33] Martin-Nieto, J., Flores, E. and Herrero, A. (1992) Plant Physiol. 100, 157–163.
[34] Ida, S. and Mikami, M. (1983) Plant Cell Physiol. 24, 649–658.
[35] Hervás, M., Navarro, J.A. and Tollin, G. (1992) Photochem. Photobiol. 56, 319–324.
[36] Wagner, R., Carillo, N., Junge, W. and Vallejos, R. (1981) FEBS Lett 131, 335–340.
[37] Batie, C.J. and Kamin, H. (1984) J. Biol. Chem. 259, 8832–8839.
[38] Batie, C.J. and Kamin, H. (1984) J. Biol. Chem. 259, 11976–11985.
[39] Winkenbach, F. and Wolk, C.P. (1973) Plant Physiol. 52, 480–483.
[40] Razquin, P., Schmitz, S., Peleato, M.L., Fillat, M.F., Gomez-Moreno, C. and Böhme, H. (1994) Photosynth. Res. 43, 35–40.
[41] Aliverti, A., Corrado, M.E. and Zanetti, G. (1994) FEBS Lett. 343, 247–250.
[42] Méndez, J.M., Herrero, A. and Vega, J.M. (1981) Z. Pflanzenphysiol. 103, 305–315.
[43] Hirasawa, M., Proske, P.A. and Knaff, D.B. (1994) Biochim. Biophys. Acta 1187, 80–88.
[44] Bosshard, H.R. (1995) in Cytochrome Source Book (Mauk, A.G. and Scott, R.A., eds.), in press, University Science Press, Mill Valley, CA.