A ferredoxin Arg-Glu pair important for efficient electron transfer between ferredoxin and ferredoxin-NADP$^+$ reductase

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Abstract In order to elucidate the importance of a ferredoxin (Fd) Arg-Glu pair involved in dynamic exchange from intra- to intermolecular salt bridges upon complex formation with ferredoxin-NADP$^+$ oxidoreductase (FNR), *Equisetum arvense* FdI and FdII were investigated as normal and the pair-lacking Fd, respectively. The FdI mutant lacking this pair was unstable and rapidly lost the [2Fe–2S] cluster. The catalytic constant (k_{cat}) of the electron transfer for FdI is 5.5 times that for FdII and the introduction of this pair into FdII resulted in the increase of k_{cat} to a level comparable to that for FdI, demonstrating directly that the Arg-Glu pair is important for efficient electron transfer between Fd and FNR.

Key words: Ferredoxin; Ferredoxin-NADP$^+$ reductase; Electron transfer; Photosynthesis; *Equisetum arvense*

1. Introduction

The electron transfer protein, [2Fe–2S] ferredoxin (Fd), accepts electrons primarily from photosystem I and donates them to Fd-NADP$^+$ reductase (FNR) catalyzing reduction of NADP$^+$ to NADPH. Fds also donate electrons to nitrite reductase, sulfite reductase and glutamate synthase in the chloroplast stroma to support the assimilation of nitrogen and sulfur.

Experimental results from chemical modification [1–4], site-directed mutagenesis [5–8] and computer modeling experiments [9] have shown that several acidic amino acid residues of Fd are involved in the interaction with FNR, and indicated that the Fd–FNR complex is stabilized by electrostatic interactions between acidic residues of Fd and basic residues of FNR. Recently, Kurisu et al. [10] determined the complete structure of the Fd–FNR complex from maize at 2.59 Å resolution. They revealed that five pairs of intermolecular salt bridges were obviously involved in the interaction between Fd and FNR in addition to a hydrophobic environment near their two prosthetic groups. Interestingly, one of these salt bridges consists of Arg40 of Fd and Glu154 of FNR in contrast to the general expectation described above. The Arg residue corresponding to Arg40 of maize Fd forms an intramolecular salt bridge with the Glu residue corresponding to Glu29 of maize Fd in the free Fd molecule [9,11–14]. Therefore, it is considered that the intramolecular salt bridge of the Arg-Glu pair is exchanged to the intermolecular salt bridge with Glu154 and Lys304 of FNR, respectively, in the process of complex formation of Fd with FNR. As the Arg is located in the cluster binding loop containing the Cys residues, which coordinate the redox-active FE1 atom in the [2Fe–2S] cluster, the recombination of the salt bridge may play an important role in the electron transfer between Fd and FNR. In fact, it is known that the redox potential of spinach Fd is negatively shifted by 90 mV in the FNR bound state [15]. However, the importance of this Arg-Glu pair in the interaction or electron transfer between Fd and FNR has not yet been demonstrated directly by biochemical studies.

Among more than 70 plant-type Fd sequences known, all but three conserve the Arg-Glu pair in question [16], suggesting their importance for the electron transfer function of Fd. The exceptional Fds are *Anabaena* 7120 heterocyst Fd [17], *Equisetum arvense* FdII [18], and *Equisetum telmateia* FdII [19]. In *Anabaena* 7120 heterocyst Fd, which is involved in the electron transfer to nitrogenase in nitrogen fixation [20], this Arg is substituted by His, and the His forms a charge–charge interaction with an Asp rather than the Glu of the Arg-Glu pair [21,22]. On the other hand, *E. arvense* and *E. telmateia* FdIIs, whose roles are not yet known, have non-charged Gln and Ser instead of Arg and Glu in the pair. The crystal structure of the other isoform, FdI from *E. arvense*, has been determined at 1.8 Å resolution [11] and it is known that this FdI contains an intramolecular salt bridge between the Arg-Glu pair (Arg39 and Glu28 in the numbering of FdI from *E. arvense*). Therefore, we considered that *E. arvense* FdI and II were favorable examples in which we elucidate the contribution of the Arg-Glu pair to the electron transfer function of Fd.

In this study, we prepared *E. arvense* FdI, FdII, a FdI mutant without the Arg-Glu pair and a FdII mutant with the Arg-Glu pair newly introduced. Their electron transfer capacities with FNR were investigated, and the affinities toward FNR were analyzed by fluorometric titrations of FNR with

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Abbreviations: DTT, dithiothreitol; Fd, ferredoxin; FNR, ferredoxin-NADP$^+$ oxidoreductase; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight

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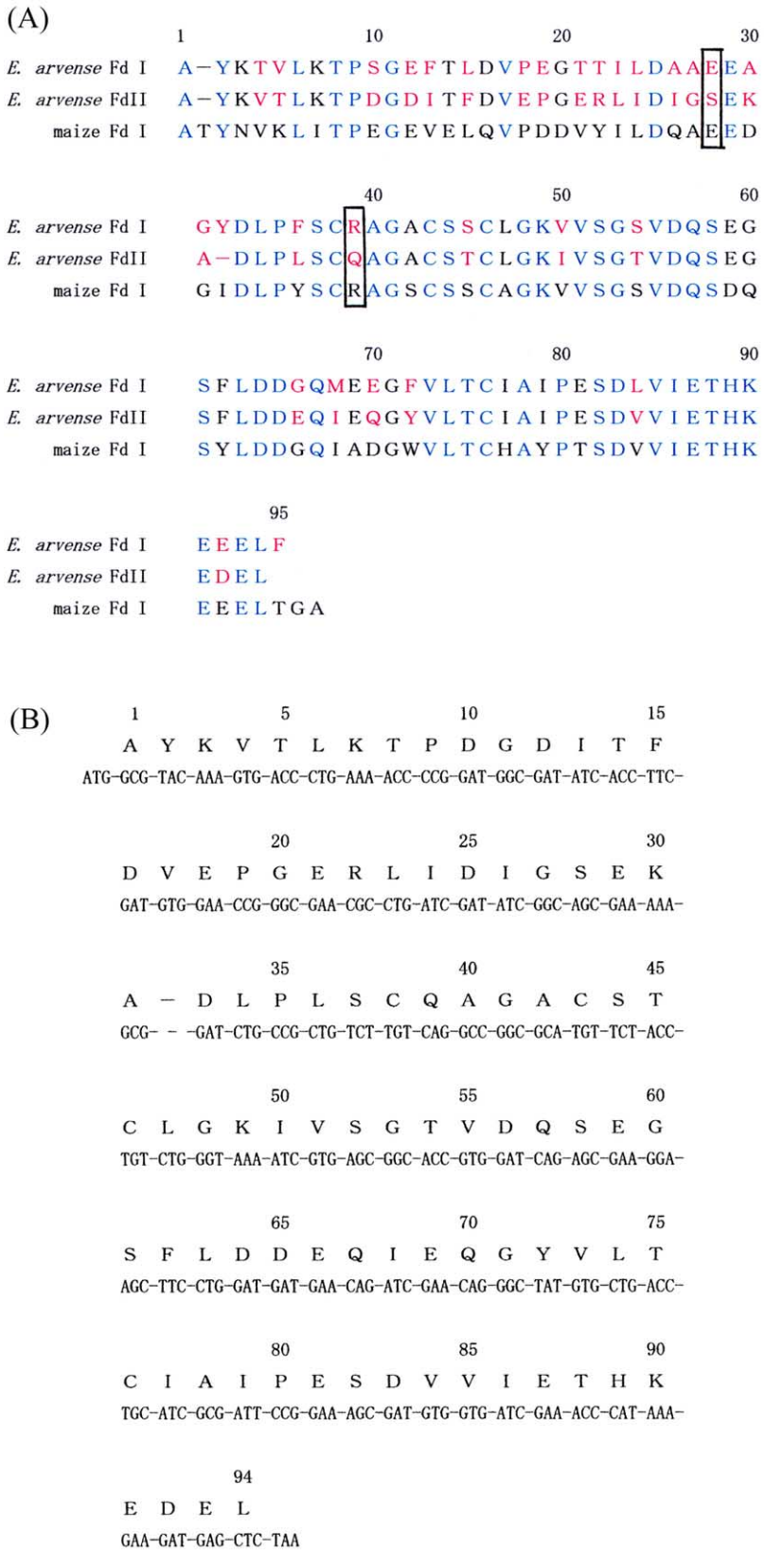

Fig. 1. A: Sequence alignment of *E. arvense* FdI, FdII [18] and maize FdI [30]. Residues different between *E. arvense* FdI and II and conserved among the three Fds are colored red and blue, respectively. The residues corresponding to the Arg-Glu pair are boxed. B: Designed DNA sequence for the mature FdII from *E. arvense*.

the Fds. We report here that the Arg-Glu pair is important for electron transfer between Fd and FNR, and furthermore this pair is crucial for determining the distinct properties of FdI and FdII from *E. arvense*.

2. Materials and methods

2.1. Construction of expression plasmids of FdI, FdII and their mutants

The cDNA of mature FdI from *E. arvense* was amplified by reverse transcription polymerase chain reaction (PCR) from young leaves of horsetail (*E. arvense*) and an approximately 300 bp PCR product was cloned in pTrc99A vector (Amersham Pharmacia Biotech). A DNA fragment encoding FdII from *E. arvense* of 93 amino acids [18] was chemically synthesized according to the method described previously [23,24]. The designed fragment (Fig. 1B) was divided into two blocks, each block of eight made-to-order oligonucleotides (Espec-oligo) was annealed and ligated, and the expression plasmid for FdII was subsequently prepared by ligation of the two separate blocks into pTrc99A vector.

Site-specific mutants of FdI R39Q/E28S, FdII Q39R/S28E and FdII D64N were prepared with the Quikchange site-directed mutagenesis kit (Stratagene). The synthetic oligonucleotides used for mutagenesis were CTACTATTCTGGATGCTGCATCAGAAGCTGGCTATGATCTTC and GAAGATCATAGCCAGCTTCTGATGCAGCATCCAGAATAGTAG for residue 28 of FdI, CTTCCGTTTAGTTGTCAGGCCGGCGCTTGCTCGAG and CTCGAAGCAAGCGCCGGCCTGACAACTAAACGGAAG for residue 39 of FdI, CGCCTGATCGATATCGGCGAGGAAAAAGCGGATCTGCCG and CGGCAGATCCGCTTTTTCCTCGCCGATATCGATCAGGCG for residue 28 of FdII, CGCTGTCTTGTCGGGCCGGCGCATG and CATGCGCCGGCCCGACAAGACAGCG for residue 39 of FdII, and GCGAAGGAAGCTTCCTGAATGATGAACAGATCG and CGATCTGTTCATCATTCAGGAAGCTTCCTTCGC for residue 64 of FdII. Underlined bases denote mismatches with the wild-type sequences.

The mutation sites and the sequence integrity of the entire coding regions of the Fds were confirmed by DNA sequencing using a dye terminator cycle sequencing kit (PE, Applied Biosystems) and an automated DNA sequencer (model 373A; Applied Biosystems).

2.2. Culture of bacterial cells, reconstitution and purification of recombinant Fds

Escherichia coli strain JM109 cells transformed with FdI, FdII and their mutant genes were grown in 3 l of LB medium with 50 μg/ml ampicillin for 3 h at 37°C, after inoculation with an overnight seed culture at 1% volume. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 0.5 mM and further cultivation was continued for 16 h. The cells harvested by centrifugation were resuspended in 50 mM Tris–HCl, pH 7.5, 60 mM NaCl, 5 mM dithiothreitol (DTT), 3 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100 and they were lysed by sonication on ice. The supernatant containing apoFd was obtained by centrifugation of the lysate at $8000 \times g$ for 15 min. Chemical reconstitution of Fd was carried out essentially according to the method described previously [25,26]. Urea (biochemical grade) was added to the supernatant to a final concentration of 8 M. Under a stream of argon gas, the solution was gently stirred for 40 min at room temperature after DTT was added to a final concentration of 100 mM. Then $FeCl_3$, $Fe(NH_4)_2(SO_4)_2$ and Na_2S were added gradually to a final concentration of 1 mM each. After further gentle stirring for 20 min under anaerobic conditions, the mixture was diluted eight-fold with degassed 50 mM Tris–HCl, pH 7.5 and 50 mM NaCl buffer. The refolded holo-Fd which assembled the [2Fe–2S] cluster was adsorbed on a DEAE-cellulose column. Further purification of Fds and their mutants was carried out as described previously [27,28]. Yields of the Fds except for FdI R39Q/E28S were about 10 mg/l. The purity of the Fds was confirmed by non-denaturing polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Ultraflex, Bruker). The observed values (calculated values) of mass for FdI, FdII, FdII Q39R/S28E, FdII D64N were 10 091 (10 097), 9964.4 (9963.1), 10 034 (10 032) and 9963.1 (9962.1), respectively. The concentration of Fd was determined spectrophotometrically based on a molar extinction coefficient of 9.68 mM^{-1} cm^{-1} at 422 nm [29].

2.3. Enzyme assays

Electron transfer ability of Fd was assayed by measuring the rate of cytochrome *c* (cyt *c*) reduction as described previously [27]. The reaction mixture contained, in a total volume of 1.0 ml, 1.0 mM NADPH, 40 nM maize leaf FNR, 0.2 mM cyt *c*, 50 mM Tris–HCl (pH 7.5) and 100 mM NaCl. The reaction was initiated by the addition of Fd at final concentrations from 0.5 to 39 μM. The reduction of cyt *c* was monitored by the increase in A_{550} at 25°C.

2.4. Fluorescence measurement

Fluorescence spectra were recorded with a Hitachi fluorescence spectrometer, F-2500. The sample solution contained 5 μM maize leaf FNR, 50 mM Tris–HCl (pH 7.5) and 0 mM or 100 mM NaCl. In order to determine K_d of Fd from the complex with FNR, fluorescence change at 338 nm (295 nm excitation) following an increase in Fd concentration from 1.0 to 12 μM was monitored. All the readings were made at 25°C. The K_d values were obtained by fitting the data to a 1:1 binding stoichiometry of Fd and FNR using a least squares method.

3. Results

The sequence alignment of *E. arvense* FdI, FdII [18] and maize FdI [30] is presented in Fig. 1A. To avoid confusion from numbering of amino acid residues in each Fd, in Sections 3 and 4 we will use the numbering for *E. arvense* FdI based on this alignment.

3.1. Preparation of recombinant wild-type and mutant Fds

Most of the Fds expressed in *E. coli* cells were accumulated as the apo-form and successfully converted to the holo-form by chemical reconstitution of the [2Fe–2S] cluster (see Section 2). The absorption spectra and the electron transfer capacities with FNR of reconstituted FdI and II were in good agreement with those of the authentic FdI and II prepared from young leaves of *E. arvense* [18] (data not shown). Only FdI R39Q/E28S, where the Arg39-Glu28 pair is substituted with the non-charged corresponding residues of FdII, was unstable and its absorption spectra due to the presence of the [2Fe–2S] cluster disappeared within 1 day, suggesting that the salt bridge between Arg39 and Glu28 of FdI is crucial for stabilizing the cluster. Note that the [2Fe–2S] cluster of FdII was stable even in the absence of a salt bridge between Arg39 and Glu28.

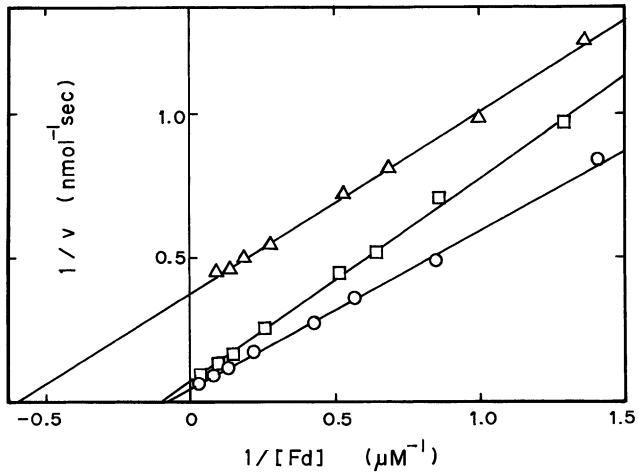

Fig. 2. Double reciprocal plots of the kinetic data for electron transfer abilities of *E. arvense* FdI (○), FdII (△), and FdII Q39R/S28E (□) with FNR in the NADPH-dependent cyt *c* reduction at 25°C, pH 7.5 and ionic strength 0.1.

3.2. Kinetics of electron transfer with FNR of Fds containing and lacking the Arg-Glu pair

The electron transfer abilities of FdI, FdII and FdII mutants were assayed in a reconstituted system of cyt *c* reduction via electron transfer from FNR to Fd in the presence of an excess of NADPH. The reciprocal velocities of cyt *c* reduction were plotted as a function of the reciprocal concentration of Fd (Fig. 2). As summarized in Table 1, the catalytic constant (k_{cat}) of FdI, which represents turnover number of electron transfer in the Fd–FNR complex, was 5.5 times that of FdII, whereas the dissociation constant (K_m) of FdII was considerably lower than that of FdI. As a result, the k_{cat}/K_m is apparently not much different between FdI and FdII. As their k_{cat} and K_m are considerably different, we suspect that the two Fd isoforms may play different roles in redox metabolic pathways.

In order to elucidate the importance of the Arg39-Glu28 pair in the electron transfer function of FdI, kinetic properties of the FdII mutant Q39R/S28E were investigated (Fig. 2). The k_{cat} and *K*m of FdII Q39R/S28E were clearly shifted from those of FdII wild-type and reached 83% and 101% of FdI values, respectively (Table 1). Therefore, the presence of the Arg39-Glu28 pair in FdI and in the FdII mutant is the major reason for the increase in their k_{cat} values compared with FdII. It was proposed that electron transfer from Fd to FNR is modulated by two intermolecular salt bridges between Arg39 of Fd and Glu154 of FNR and between Glu28 of Fd and Lys304 of FNR in the maize Fd–FNR complex [10] and this seems to be the case for FdI of *E. arvense*.

FdII has higher affinity toward FNR than FdI and FdII Q39R/S28E in spite of lacking the Arg-Glu pair, suggesting that the binding mode of FdII to FNR may differ considerably from that of Fd containing the Arg-Glu pair. The particular importance of Asp64, among five residues of Fd participating in the intermolecular salt bridges in the maize Fd–FNR complex, has been indicated by site-directed mutagenesis of maize Fd isoforms [7]. In order to elucidate the importance of Asp64 in the unique interaction of *E. arvense* FdII with FNR, we also investigated the catalytic ability of FdII D64N. As shown in Table 1, the affinity of FdII D64N toward FNR decreased about one third for FdII wild-type and this result was comparable with the data shown by mutagenesis experiments with maize Fds [7]. Therefore, we suspect that the interaction of FdII with FNR contains at least an intermolecular salt bridge between Asp64 of Fd and Lys91 of FNR as indicated by the crystallographic study [10].

3.3. Dissociation constants of the Fds from Fd–FNR complexes

Fig. 3A shows fluorescence spectra of FNR measured in the presence and absence of Fd under various salt conditions. The tryptophyl fluorescence intensity of FNR excited at 295 nm markedly decreased on addition of either FdI or FdII, both of which contain no Trp residue. The crystallographic data indicated that Trp296 and Trp309 of FNR are located in close proximity to the interface of the Fd–FNR complex [10] and the surrounding environment of the Trp residue(s) may be perturbed upon interaction with Fd. The difference in fluorescence intensity at 338 nm was plotted as a function of the molar concentration of free Fds as shown in Fig. 3B. The data were analyzed on the assumption that binding of Fd to FNR obeys a 1:1 stoichiometry.

As listed in Table 2, K_ds of FdI and FdII are comparable with each other at 0 mM NaCl (very low ionic strength), while at 100 mM NaCl, the K_d of FdI is 1.8 times that of FdII. K_ds of FdI and FdII are 2.9 and 1.9 times greater respectively than

Table 1
Kinetic parameters of FdI, FdII, and FdII mutants for electron transfer reaction with FNR in the NADPH-dependent cyt *c* reduction

Fd species	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
Wild-type FdI	11.4 ± 0.98	177 ± 13	15.5 ± 0.22
Wild-type FdII	1.66 ± 0.11	32.2 ± 2.4	19.3 ± 0.27
FdII Q39R/S28E	11.5 ± 0.68	147 ± 6.3	12.8 ± 0.23
FdII D64N	5.26 ± 0.21	36.8 ± 1.7	7.01 ± 0.05

These data were extracted from the Fd saturation curves. K_m and k_{cat} for Fds were determined at 25°C, pH 7.5 and 100 mM NaCl from a double reciprocal plot. The values are means ± S.D. of three independent determinations.

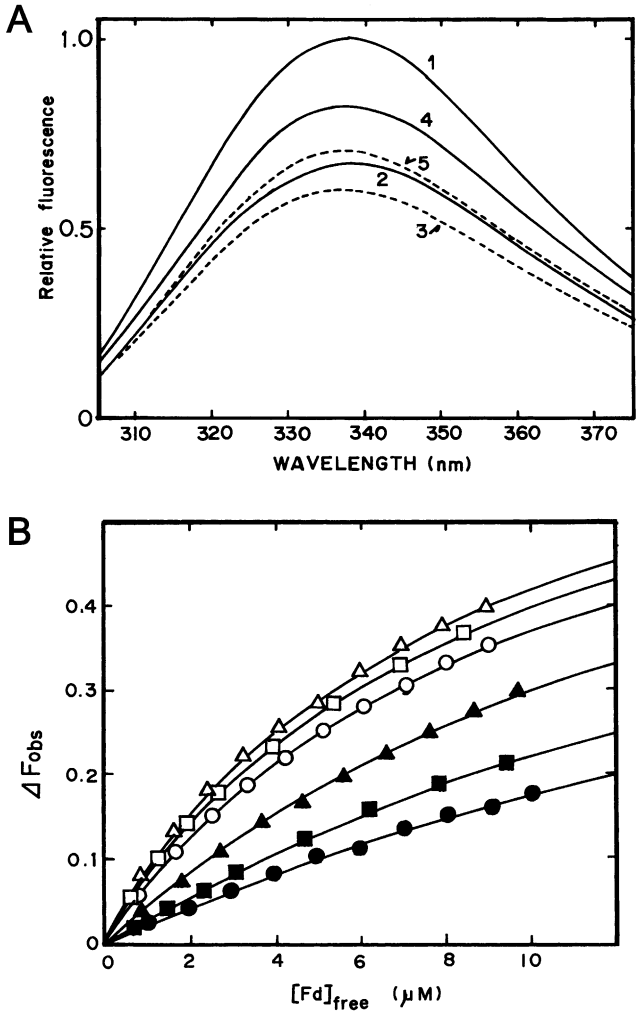

Fig. 3. Determination of dissociation constants (K_d) of *E. arvense* FdI, FdII, and FdII Q39R/S28E from the respective complexes with FNR at 25°C and pH 7.5. A: Tryptophyl fluorescence spectra of FNR in the absence and presence of Fd. 1, in the absence of Fd at 0 M NaCl. Its spectrum at 100 mM NaCl was not changed; 2, in the presence of 11.4 μM FdI at 0 M NaCl; 3, 11.6 μM FdII at 0 M NaCl; 4, 11.4 μM FdI at 100 mM NaCl; 5, 11.6 μM FdII at 100 mM NaCl. B: Difference fluorescence intensities at 338 nm of FNR, plotted as a function of the molar concentrations of FdI (circles), FdII (triangles) and FdII Q39R/S28E (squares). Open and filled symbols indicate the values at 0 M and 100 mM NaCl, respectively. The solid curves are the theoretical ones.

at 0 mM NaCl. Therefore, the interaction of FdI with FNR is more affected by the ionic strength than that of FdII. This phenomenon is compatible with the crystallographic finding that each residue of the Arg-Glu pair in Fd forms an intermolecular salt bridge with an FNR residue of the opposite charge.

The K_ms of FdI and FdII obtained by measuring kinetic abilities of the Fds at 100 mM NaCl (Table 1) are 2.6 and 9.6 times smaller than the respective K_ds at the same ionic strength (Table 2). The K_d indicates the dissociation constant between oxidized Fd and oxidized FNR, while the K_m indicates the dissociation constant between oxidized Fd and reduced FNR with an excess of NADPH. Therefore, the affinity of Fd may be much higher for reduced FNR, or the reduced FNR–NADPH complex, than for oxidized FNR.

As listed in Table 2, the K_d of FdII Q39R/S28E is comparable with the K_ds of FdI and FdII at 0 mM NaCl, while the K_d of FdII Q39R/S28E is shifted near to that of FdI rather than FdII at 100 mM NaCl. This finding is compatible with the observations of K_m. We therefore suspect that Arg39 and Glu28, introduced into FdII, preferentially form intermolecular salt bridges with the counter residues of FNR doing complex formation and hence any unique interaction of FdII with FNR, probably containing several hydrogen bondings and hydrophobic interactions, could not be achieved.

As also shown in Table 2, the affinity of FdII D64N toward FNR decreased about half for FdII wild-type and this result was compatible with the corresponding kinetic data.

4. Discussion

The presented data of FdI, FdII and FdII Q39R/S28E demonstrate well the importance of the Arg-Glu pair for efficient electron transfer between the two proteins in the Fd–FNR complex, suggesting that recombination of the salt bridge from an intra- to an intermolecular one produces a transition state favorable for intermolecular electron transfer, due to the relative configuration of Fd and FNR or redox potential shifts of the prosthetic groups. As the FdI mutant lacking the Arg-Glu pair became unable to maintain the [2Fe–2S] cluster, the two intermolecular salt bridges might also contribute to stabilizing the cluster of Fd in the Fd–FNR complex.

The kinetic parameters (K_m and k_{cat}) of FdI and FdII were found to be obviously different from each other. FdII has a low k_{cat} value but a high affinity toward reduced FNR compared with FdI. Introduction of only the Arg-Glu pair into FdII shifted the values of its kinetic parameters to nearly equal those of FdI, although the amino acid sequence of FdII differs at 30 sites from that of FdI (Fig. 1A). Therefore, it is concluded that the Arg-Glu pair is crucial for determining the distinct properties of FdI and FdII from *E. arvense*.

Table 2
Dissociation constants (K_d) of FdI, FdII and FdII mutants from the complexes with FNR

Fd species	K_d (μM) at 0 mM NaCl	K_d (μM) at 100 mM NaCl
Wild-type FdI	9.90 ± 0.75	28.6 ± 2.4
Wild-type FdII	8.19 ± 0.64	15.9 ± 0.68
FdII Q39R/S28E	9.12 ± 0.9	24.7 ± 1.7
FdII D64N	18.9 ± 1.4	29.2 ± 2.3

K_ds for Fds were determined from the titration data of tryptophyl fluorescence of FNR with Fds at 25°C and pH 7.5. The values are means ± S.D. of three independent determinations.

We are currently studying the X-ray structure of *E. arvense* FdII and the preliminary data show that Glu59, involved in the intermolecular salt bridge in the maize Fd–FNR complex, forms an intramolecular salt bridge with Arg22. Consequently, Glu59 of FdII is masked in complex formation with FNR. In addition to the absence of Arg39 and Glu28, this suggests that the binding mode of FdII to FNR is considerably different from that of FdI, although the data of FdII D64N indicated that the interaction of FdII with FNR contains at least another intermolecular salt bridge consisting of Asp64 of Fd and Lys91 of FNR [10]. After the complete analysis of the FdII structure, we will investigate the relation of redox potentials of FdI and FdII with their environments around the [2Fe–2S] cluster, and explore how the Arg-Glu salt bridge is involved in the redox regulation.

There are generally more than two isoforms of Fd in each species of plant, and Fds donate electrons to several Fd-dependent enzymes and appear to participate in not only assimilation of inorganic substances, but also regulation of Calvin cycle enzymes and cyclic photophosphorylation [31]. Therefore, it is very interesting to speculate what role FdII plays in these redox metabolic pathways.

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