Problem Set 7 (**Due March 11th**)

1. Using the KEGG Pathways website, navigate to the Photosynthesis page. You should see a cartoon image of all the relevant proteins and complexes.
    a. What is the name and structure of the quinone that carries electrons from PSII to Cyt b_6f? How is this different than the quinone that is important in mitochondrial electron transport?
    b. For every NADPH that is produced in the stroma, how many protons are produced in the lumen?
    c. How many protons are required to synthesize 1 ATP?
    d. Click on the Antenna Proteins link: How many light harvesting proteins are associated with each photosystem? What are their names?

2. Describe the four fates of excited electrons and indicate which are important for photosynthesis. Justify your answer.

3. What is the difference between Chlorophyll A, Chlorophyll B, and pheophytin?

4. As we discussed in class, there are roughly 300 chlorophyll molecules for every photosynthetic reaction center. Noting that not all of these chromophores are part of the reaction center, what is the role of these additional molecules? Please discuss the proteins that harbor these additional chloryphyll molecules and how your answer to problem 1 is relevant.

5. The Z scheme and the red drop are two important features of photosynthesis. Describe what is meant by each of these terms. In addition, please describe an experiment that uses the red drop to confirm the Z scheme. (include a sketch of the data and explain what it means).

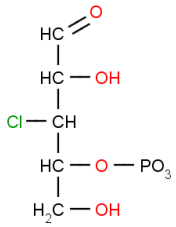
6. Energy and efficiency:
    a. How many photons of red light (λ = 650 nm) are needed to produce 1 O_2 from 2 H_2O?
    b. Noting that it takes 8 photons to carry out this process, how efficient is photosynthesis?
    c. So clearly energy is lost in this process. Propose a reason for the energy loss.
    d. For every O_2 that is produced at the OEC, 12 protons are accumulated in the thylakoid lumen. Account for all of these protons (problem 1 should help with this)
    e. Calculate how many ATP are produced per photon of red light (λ = 650 nm) – remember to account for the efficiency that you determined in 5b.
    f. Noting that it takes 30.5 kJ mol^{-1} to make ATP from ADP and Pi, how many photons (λ = 650 nm) should be theoretically needed?

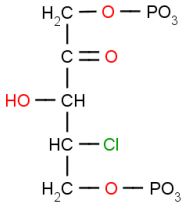
7. Photosynthetic Reaction Center:
    a. Using the crystal structure of the photosynthetic reaction center from *Rps. viridis*, (pdbID 1PRC), make an image that shows just the redox cofactors (you should be able to make it resemble Figure 29-57 in your book). Color the Special Pair red, menaquinone black, ubiquinone blue and the pheophytins green.
    b. What is the distance between Mg^{2+} ions in the special pair? What is the reason that these two chlorophyll molecules are so close?
    c. Please show the sequence of electron transfer steps in this reaction center and list the approximate times it takes for each step.
    d. Recalling that fluorescence and internal conversion are very slow processes (~ 200μs), what is the significance of the rate of electron transfer in the scheme you determined in 5c?

e. What is the source of electrons that fill the hole that is formed upon photon absorption in PSI and PSII?
f. What is the fate of electron that is excited at PSII?

8. Draw a reaction mechanism for the carbon fixation step of the Calvin Cycle.

9. Predict the product of a transketolase reaction between the two molecules to the right.





10. As noted in class, the Calvin Cycle image shown in the lecture slides contains an error in the 2nd transketolase reaction (S7P + GAP).
    a. Show the mechanism of this reaction and determine what the products should be.
    b. The aldose that is produced in this reaction can be converted directly to Ru5P in a mechanism that is identical to one of the steps in glycolysis. Identify the enzyme that catalyzes this transformation and predict a mechanism.

11. Triose phosphates are the direct product of the Calvin Cycle
    a. Which triose phosphates are produced?
    b. How can these compounds be converted to hexose phosphates and pentose phosphates?

12. Read the manuscript attached to answer the following questions:
    a. Ferredoxin is important in a wide variety of cellular processes including carbon assimilation, nitrogen assimilation, sulfur assimilation and cellular redox control. It participates in these processes through interactions with a variety of proteins. For each of the pathways listed above, identify one or more partner proteins. Which of these interactions is important in photosynthesis?
    b. Describe the goals of this study.
    c. What amino acids were mutated in this study and why?
    d. How did the investigators confirm that site-directed mutants don't significantly perturb protein structure?
    e. Figure 5 confirms that Fd and SiR form a complex at pH 7.5. Describe how this conclusion was reached based on the data.
    f. Compare the results from Figure 6 to the activity data from Table 2. Describe any correlation observed between these two pieces of experimental data.

ENZYMOLOGY:

Comparison of the Electrostatic Binding Sites on the Surface of Ferredoxin for Two Ferredoxin-dependent Enzymes, Ferredoxin-NADP $^{+}$ Reductase and Sulfite Reductase

Tetsuyuki Akashi, Tomohiro Matsumura, Takashi Ideguchi, Ken-ichiro Iwakiri, Takenobu Kawakatsu, Isao Taniguchi and Toshiharu Hase

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Comparison of the Electrostatic Binding Sites on the Surface of Ferredoxin for Two Ferredoxin-dependent Enzymes, Ferredoxin-$NADP^+$ Reductase and Sulfite Reductase*

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Plant-type ferredoxin (Fd), a [2Fe-2S] iron-sulfur protein, functions as an one-electron donor to Fd-$NADP^+$ reductase (FNR) or sulfite reductase (SiR), interacting electrostatically with them. In order to understand the protein-protein interaction between Fd and these two different enzymes, 10 acidic surface residues in maize Fd (isoform III), Asp-27, Glu-30, Asp-58, Asp-61, Asp-66/Asp-67, Glu-71/Glu-72, Asp-85, and Glu-93, were substituted with the corresponding amide residues by site-directed mutagenesis. The redox potentials of the mutated Fds were not markedly changed, except for E93Q, the redox potential of which was more positive by 67 mV than that of the wild type. Kinetic experiments showed that the mutations at Asp-66/Asp-67 and Glu-93 significantly affected electron transfer to the two enzymes. Interestingly, D66N/D67N was less efficient in the reaction with FNR than E93Q, whereas this relationship was reversed in the reaction with SiR. The static interaction of the mutant Fds with each the two enzymes was analyzed by gel filtration of a mixture of Fd and each enzyme, and by affinity chromatography on Fd-immobilized resins. The contributions of Asp-66/Asp-67 and Glu-93 were found to be most important for the binding to FNR and SiR, respectively, in accordance with the kinetic data. These results allowed us to map the acidic regions of Fd required for electron transfer and for binding to FNR and SiR and demonstrate that the interaction sites for the two enzymes are at least partly distinct.

Plant-type ferredoxin (Fd)[1] is a small (11-kDa), soluble, acidic protein distributed in plants, algae, and cyanobacteria. This protein contains a single [2Fe-2S] cluster and its oxidation-reduction potential is very low ranging from −310 mV to −455 mV (1). Amino acid sequences of more than 70 plant-type Fds are highly homologous (2). X-ray crystallographic structures of five plant-type Fds from cyanobacteria (3–7) to higher plants (8) are also conserved in term of backbone and side-chain structures.

In chloroplasts, this type of Fd mediates one-electron transfer from photosystem I to several Fd-dependent enzymes, which function in photosynthetic metabolism, such as ferredoxin-$NADP^+$ reductase (FNR) (EC 1.18.1.2), which is involved in the process of carbon assimilation; nitrite reductase and glutamate synthase, which are involved in nitrogen assimilation; sulfite reductase (SiR) (EC 1.8.7.1), which is involved in sulfur assimilation; and ferredoxin-thioredoxin reductase, which is involved in redox regulation of several enzymes (9). Fd and each Fd-dependent enzyme form a 1:1 protein-protein complex, and this specific interaction is considered to be important for efficient electron transfer between the two proteins. The sites involved in the interaction between Fd and its complementary electron transfer partners have been studied in several laboratories, although the actual geometry of the complex has not been established. Among the physical and chemical forces involved in protein interactions, such as hydrophobic packing interaction, electrostatic forces, and hydrogen bonding, several lines of evidence from chemical modification experiments (10–16), cross-linking experiments (17, 18), and computer modeling studies (3, 19) indicate that the complex is mainly formed as a result of electrostatic forces through the negative charges of Fd and the positive charges of each enzyme. Ionic strength affects the transient kinetics of electron transfer from Fd to FNR also indicating that complementary electrostatic charges influence complex formation (20).

In this study, we attempted to identify and compare the binding sites in Fd for two Fd-dependent enzymes, FNR and SiR, by site-directed mutagenesis of maize Fd (isoform III) (21, 22). FNR is a 35-kDa, soluble flavoprotein containing one non-covalently bound flavin adenine dinucleotide, and it catalyzes the reduction of $NADP^+$ to NADPH with two electrons from reduced Fd. SiR found in higher plants is a 64-kDa, soluble protein containing one [4Fe-4S] cluster and one siroheme, and it catalyzes the six-electron reduction of sulfite to sulfide. Acidic residues (Asp and Glu) located at eight different sites in maize Fd, Asp-27, Glu-30, Asp-58, Asp-61, Asp-66/Asp-67, Glu-71/Glu-72, Asp-85, and Glu-93 were chosen as targets of substitution, to be replaced by the corresponding amide residues. Although the three-dimensional structure of maize Fd is not known, the structure could be superimposed on those of the three plant-type Fds (Fig. 1), because about 60–70% of the amino acid sequence of maize Fd is identical to those of the three Fds. The spatial orientation of the side-chains of the acidic residues corresponding to those altered through mutagenesis in maize Fd is considered to be similar among the plant-type Fds (Fig. 1). We examined the ability of each of the resulting Fd mutants to bind to FNR and SiR and their capacity for electron transfer. The binding ability was successfully

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[1] The abbreviations used are: Fd, ferredoxin; FNR, ferredoxin-$NADP^+$ reductase; SiR, sulfite reductase.

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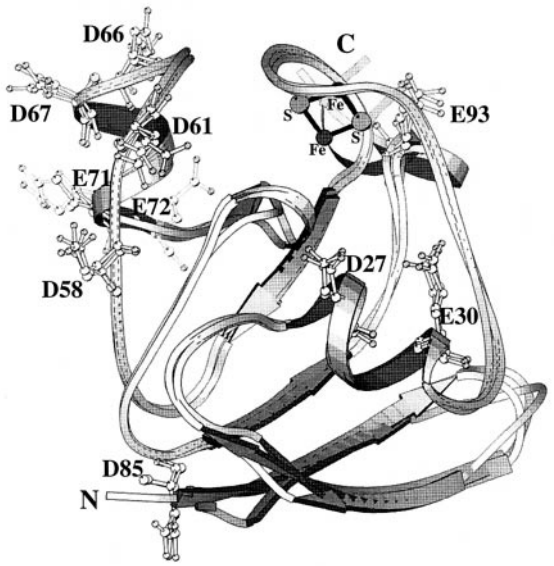

FIG. 1. **The three-dimensional structures of plant-type Fds.** The backbones of the three plant-type Fds from *Spirulina*, *Equisetum*, and *Anabaena* are superimposed. Side-chains of 10 conserved acidic residues are shown with ball and stick style. The numbering of the acidic residues corresponds to that of maize Fd (isoform III). The [2Fe-2S] cluster located on the edge of the molecule is also shown. Amino- and carboxyl termini are marked *N* and *C,* respectively.

analyzed by gel filtration of a mixture of Fd and each enzyme. Affinity chromatography on Fd-immobilized resins was also applied to evaluate the interaction of the two proteins. We report here that certain acidic residues are indeed crucial for the interaction with FNR and SiR and furthermore that the distribution of such residues in the three-dimensional structure of Fd is partly distinct for the two enzymes.

EXPERIMENTAL PROCEDURES

Mutagenesis of the Fd Gene—The insert DNA in the region from the *Nco*I site to the *Xho*I site of pSMmFD3, a maize Fd III expression plasmid (22) originally constructed using the vector pKK233–2 (Amersham Pharmacia Biotech), was ligated into the *Nco*I/*Xho*I cloning site of another expression vector, pTrc99A (Amersham Pharmacia Biotech) to obtain pSMmFD3–1. For construction of D66N/D67N and D66K/D67K, cassettes of two complementary oligonucleotides (Table I) were inserted into the *Bam*HI and *Pst*I sites of pSMmFD3–1. Other mutant Fds were constructed by an overlap extension method by two-step polymerase chain reaction (23, 24), using a combination of two terminal primers and a pair of two mutagenic primers as listed in Table I. The terminal primers were designed to produce *Nco*I and *Xho*I sites at the ends of each amplified fragment, and the fragments with the mutation site were easily inserted into the corresponding region of pSMmFD3–1. All mutation sites and the sequence integrity of the entire coding region of Fd were confirmed by DNA sequencing using a dye terminator cycle sequencing kit (Applied Biosystems) and an automated DNA sequencer (model 370A; Applied Biosystems).

Culture of Bacterial Cells and Preparation of Recombinant Fds—*Escherichia coli* strain JM105 cells transformed with various mutant Fd genes were grown in 8 liters of Luria broth medium supplemented with 100 μM $FeSO_4$ and 50 μg/ml ampicillin for 2 h at 37 °C after inoculation with an overnight seed culture at 1% volume. Isopropyl-β-D(−)-thiogalactoside was then added to a final concentration of 0.5 mM, and cultivation was continued for a further 8–12 h. The cells were harvested by centrifugation at 3000 × *g* for 10 min and stored at −30 °C until use.

Fd was extracted and purified essentially according to a published method (22, 25). The purity of the Fds was checked by nondenaturing polyacrylamide gel electrophoresis using a gel with a linear gradient of 15–25% acrylamide as described previously (26). The concentration of Fd was determined spectrophotometrically based on a molar extinction coefficient of 9.68 mM^{-1} cm^{-1} at 422 nm (27).

Preparation of FNR and SiR from Maize Leaves—Maize leaves were broken into a fine powder in liquid nitrogen with a Waring blender and homogenized in an extraction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% (v/v) 2-mercaptoethanol) with 10% (w/v) Polyclar AT. Thereafter, the homogenate was roughly filtered through several layers of cheesecloth, and the leaf extract was fractionated by ammonium sulfate precipitation. FNR and SiR were recovered in the fraction from 40–70% saturation and were separated by chromatography on DE-52 (Whatman), developed with a linear gradient of NaCl from 0 to 400 mM in 50 mM Tris-HCl, pH 7.5. Both enzymes were separately purified by successive chromatographic steps on columns of Sephacryl S-200, Blue-Sepharose (Amersham Pharmacia Biotech), and Fd-immobilized resin essentially according to published procedures (28).

The concentrations of FNR and SiR were determined spectrophotometrically based on molar extinction coefficients of 9.40 mM^{-1} cm^{-1} at 459 nm (29) and 18.0 mM^{-1} cm^{-1} at 587 nm (30), respectively.

Cyclic Voltammetry—Cyclic voltammetry of Fds was carried out using a BAS-50W electrochemical analyzer with a poly-L-lysine modified In_2O_3 electrode as described previously (31). Fds were dissolved in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl at a concentration of 50 μM, and voltammetric responses were measured at a scan rate of 2 mV/s under anaerobic conditions, purged with nitrogen gas. Catalytic reactions of FNR and SiR through the reduction of Fds on the electrode were measured in a mixture consisting of 0.25 μM FNR or SiR, 50 μM Fd in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl with 0.3 mM $NADP^+$ or 0.6 mM Na_2SO_3, respectively, as the substrate.

CD Spectrum—CD spectra of mutant Fds were measured with a

TABLE I

A list of oligonucleotides used for site-directed mutagenesis

Italic letters show the nucleotide changed by mutagenesis. The underlined nucleotide sequences indicate restriction sites.

Product		Oligonucleotide
		Cassette
		N N
D66N/D67N	5′	GATCCTTCCTT*AA*T*A*ACGGTCTGCA 5′
	3′	GAAGGAA*TT*A*T*TGCCAG 3′
		*Bam*H1 *Pst*I
		K K
D66K/D67K	5′	GATCCTTCCTT*AAG**AAA*GGTCTGCA 5′
	3′	GAAGGAA*TTC**TT**T*CCAG 3′
		*Bam*H1 *Pst*I
		primer
		N
D27N	5′	ACATCCTT*A*ACGCTGCCGA 3′
	3′	GATGTAGGAA*TT*GCGACGGC 5′
		Q
E30Q	5′	GACGCTGCC*C*AGACTGCCGG 3′
	3′	CTGCGACGGG*G*TCTGACGGCCACACC 5′
		N
D58N	5′	GGTTCGGTT*A*ACCAGTCGG 3′
	3′	CCAAGCCAA*TT*GGTCAGCC 5′
		N
D61N	5′	GACCAGTCG*A*ATGGGTCCTT 3′
	3′	CTGGTCAGC*TT*ACCCAGGAAGGAAC 5′
		Q Q
E71Q/E72Q	5′	GACGGGCTG*C**AG**C*AGCAAGGTTATGT 3′
	3′	GCCCGAC*G*TC*G*TCGTTCCAA 5′
		N
D85N	5′	ACCCAAAGTCC*A*ACTGCGTCA 3′
	3′	GGGTTTCAGG*TT*GACGCAG 5′
		Q
E93Q	5′	CCACACCCACAAG*C*AAGGCGACCT 3′
	3′	GTGGGTGTTC*G*TTCCGCTGG 5′
	5′	AAACAGACCATGGCTGTATA 3′
Terminal		*Nca*I
	3′	TTTACGAGAGCTCACC 5′
		*Xho*I

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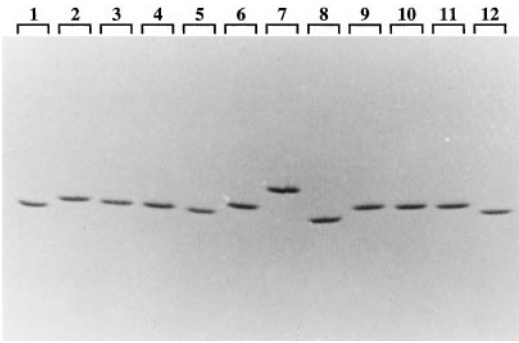

FIG. 2. **Comparison of the electrophoretic mobilities of wild type and mutated Fds on nondenaturing polyacrylamide gel.** *Lanes 1, 8,* and *12,* wild type Fd; *lane 2,* D27N; *lane 3,* E30Q; *lane 4,* D58N; *lane 5,* D61N; *lane 6,* D66N/D67N; *lane 7,* D66K/D67K; *lane 9,* E71Q/E72Q; *lane 10,* D85N; *lane 11,* E93Q.

JASCO J-720 spectropolarimeter. Fd was dissolved in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl at a final concentration of 45 and 4.5 μM for the measurements in the visible and UV regions, respectively.

Gel Filtration Chromatography—Complex formation between Fd and SiR and between Fd and FNR was analyzed by gel filtration chromatography using the Smart system with a μpeak Monitor (Amersham Pharmacia Biotech). A mixture (30 μl) of Fd and SiR at certain concentrations was loaded on a small column of Superdex 75 (PC3.2/30; Amersham Pharmacia Biotech) and eluted with 50 mM Tris-HCl, pH 7.5, 10 mM NaCl at a constant flow rate of 40 μl/min at 15 °C. Fd and SiR were monitored by the absorbance at 330 nm derived from the prosthetic groups of the proteins. For chromatography of the mixture of Fd and FNR, all conditions were the same as above except that 10 mM NaCl was omitted from the elution buffer.

Affinity Chromatography—One mg of Fd was immobilized on 0.5 g of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the method recommended by the supplier. A small column (HR 5/2; Amersham Pharmacia Biotech) packed with 200 μl of the Fd-immobilized resin was mounted in the Smart system. After equilibrating the column with 50 mM Tris-HCl, pH 7.5, 30 μl of 1.5 μM FNR or SiR was loaded and a linear gradient of NaCl from 0 to 400 mM was applied as eluent at a flow rate of 50 μl/min. Elution of the enzymes was monitored by the absorbance at 280 nm.

Enzyme Assays—FNR activity was assayed by monitoring the photoreduction of $NADP^+$ with thylakoid membranes of spinach chloroplasts in the presence of various concentrations of Fd (0–40 μM) essentially according to the method described previously (22). SiR activity was assayed by monitoring the coupling reaction with cysteine synthase according to a published method (28). Briefly, the reaction mixture consisted of 100 mM HEPES-NaOH, pH 7.5, 5 mM dithiothreitol, 0.5 mM Na_2SO_3, 12.5 mM *O*-acetyl-L-serine, an excess amount of cysteine synthase, 20 nM SiR, and various concentrations of Fds (0–10 μM). The reaction was started by addition of $Na_2S_2O_4$ at a final concentration of 8 mM, and the amount of cysteine formed in 30 min at 30 °C was determined by an acid-ninhydrin reaction.

RESULTS

Preparation of Mutant Fds—All mutant Fds isolated from *E. coli* cells were assembled with the [2Fe-2S] cluster and showed absorption spectra comparable to that of wild type Fd, with the value of the A_{422}/A_{276} ratio being greater than 0.48 (data not shown). Each migrated as a single band during nondenaturing polyacrylamide gel electrophoresis, and their mobilities were slower than that of the wild type Fd in accordance with their net charge differences, suggesting that the mobility shift was mainly due to the surface charge change introduced by mutation (Fig. 2).

Redox Potential and CD Spectra—The redox potentials of the wild type and mutant Fds were measured by cyclic voltammetry (Table II). The wild type Fd had a redox potential of −321 mV (*versus* a normal hydrogen electrode), and only the value in the case of E93Q was shifted to a significantly higher value by 67 mV. All other mutations had little effect on the redox potential. The shift in the case of E93Q seemed not to be due to any large conformational change in the backbone or any structural perturbation of the cluster, because the CD spectra of E93Q in both the ultra-violet and visible regions were essentially the same as those of the wild type Fd and D66N/D67N as shown in Fig. 3.

TABLE II

Electron transfer activity of wild type and mutated Fds in interaction with FNR and SiR and the redox potential of these Fds

Electron transfer activity was measured with 10 μM Fd as an electron donor in the present of FNR or SiR under the conditions described under "Experimental Procedures," and relative values for mutated Fds are shown with the level of activity displayed by the wild type Fd taken as 100%. The redox potential (E_m) values given are those obtained against a normal hydrogen electrode (NHE). The numbers in parentheses are differences from the E_m values of wild type Fd.

Fd form	Activity of		E_m
	FNR	SiR	
	%		*mV (δ)*
Wild type	100	100	−321
D27N	122	95	−316 (+5)
E30Q	100	81	−324 (−3)
D58N	92	102	−317 (+4)
D61N	84	88	−321 (0)
D66N/D67N	31	69	−325 (−4)
D66K/D67K	16	64	−326 (−5)
E71Q/E72Q	94	117	−321 (0)
D85N	105	98	−323 (−2)
E93Q	50	34	−254 (+67)

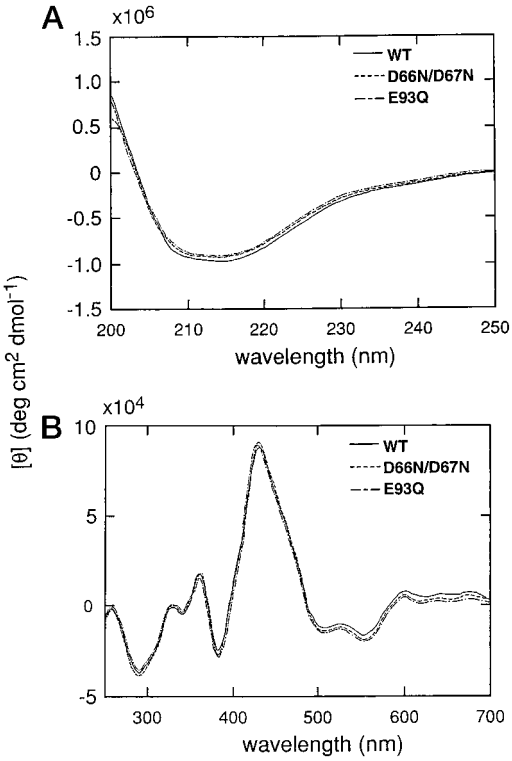

FIG. 3. **Circular dichroism spectra of wild type and mutated Fds.** Far UV spectra (*A*) and visible spectra (*B*) of wild type (*WT*), D66N/D67N, and E93Q are shown. 45 and 4.5 μM Fd was dissolved in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl for the measurements in the visible and UV regions, respectively.

Biochemical Assay of Electron Transfer—Mutant Fds were tested to assess the ability to transfer electrons to two different Fd-dependent enzymes, FNR and SiR. As summarized in Table II, significant variation in electron transfer ability was found. Among all of the mutant Fds, the mutations at Asp-66/Asp-67 and Glu-93 caused the largest decrease in electron transfer to

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TABLE III
Kinetic parameter values for FNR and SiR in interaction with wild type Fd, or the mutant Fd, D66N/D67N, or E93Q

Fd form	FNR		SiR	
	K_m	V_{max}	K_m	V_{max}
	μM	μmol/min · mg chl	μM	μmol/min
Wild type	1.7[a]	1.3[a]	1.7[a]	2.6[a]
D66N/D67N	7.3[b]	0.7[b]	2.0[a]	1.9[a]
E93Q	4.0[b]	0.6[b]	4.2[a]	1.0[a]

[a] A maximum of 10 μM Fd was used in the assay.
[b] A maximum of 40 μM Fd was used in the assay.

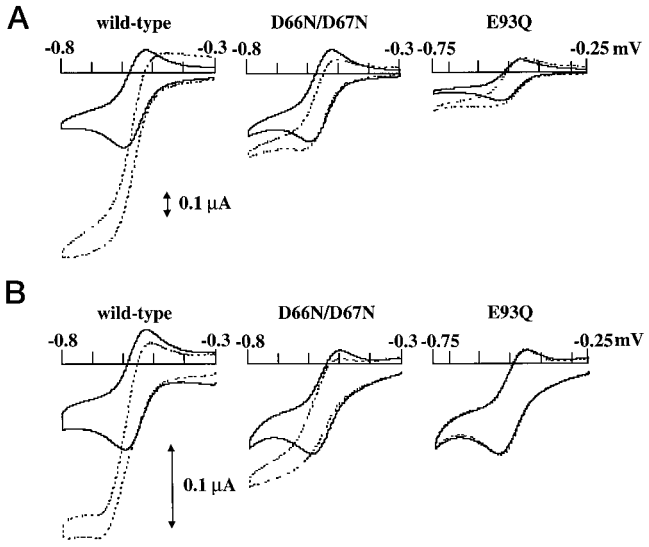

FIG. 4. **Assay of electron transfer from Fd to FNR and SiR by cyclic voltammetry.** *A,* cyclic voltammograms obtained using reaction mixtures containing 50 μM Fd and 0.25 μM FNR without (*solid line*) or with (*dashed line*) 0.3 mM $NADP^+$. *B,* cyclic voltammograms obtained using reaction mixtures containing 50 μM Fd and 0.25 μM SiR without (*solid line*) or with (*dashed line*) 0.6 mM Na_2SO_3.

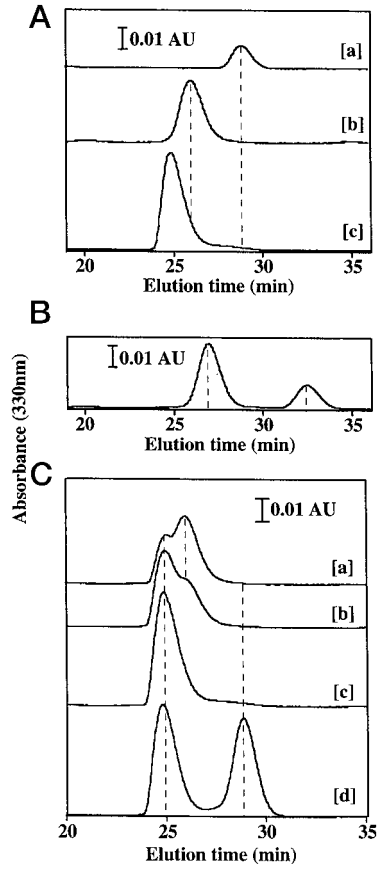

FIG. 5. **The interaction between Fd and SiR demonstrated by gel filtration chromatography on a Superdex 75 column.** *A,* elution profiles of 30 μl of 3 μM Fd (*a*), 3 μM SiR (*b*), and the mixture of the two proteins (*c*) when developed with 50 mM Tris-HCl, pH 7.5. *B,* elution profile of the mixture of the two proteins when developed with 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl. *C,* titration of Fd concentrations for binding to SiR: 30 μl of 3 μM SiR was mixed with 30 μl of 0.75 μM (*a*), 1.5 μM (*b*), 3.0 μM (*c*), and 12 μM (*d*) Fd.

FNR and SiR, respectively. It is noteworthy that mutations in two acidic regions had different effects on the electron transfer activity; D66N/D67N and D66K/D67K each had lower activity in electron transfer to FNR than in electron transfer to SiR, whereas E93Q had the reverse effect. Kinetic analyses revealed that the mutations caused an increase in K_m values and a decrease in V_{max} values (Table III). The subtle difference in K_m values between the two mutant Fds implies that Asp-66/Asp-67 and Glu-93 may differentially contribute to the affinity of Fd for FNR and SiR.

Electrochemical Assay of Electron Transfer—In addition to the biochemical assay of the mutant Fds, electron transfer from D66N/D67N and E93Q to FNR and SiR was measured by cyclic voltammetry. As shown in Fig. 4, a catalytic reduction current was observed due to the continuous oxidation of the wild type Fd in the presence of FNR and $NADP^+$, or SiR and sulfite. This catalytic current was largely decreased when either D66N/D67N or E93Q was used as an electron carrier from the electrode to the enzymes. In the case of SiR, the extent of the decrease was found to be larger with E93Q than with D66N/D67N, in agreement with the results obtained in the biochemical assay as described above.

Electrostatic Binding of Mutant Fds to SiR and FNR—When a mixture of Fd and SiR in a molar ratio of 1:1 was loaded onto a Superdex G-75 column equilibrated and developed with 50 mM Tris-HCl, pH 7.5, these proteins were eluted as a single peak at a retention time earlier than that of Fd or SiR applied singly (Fig. 5*A*). Upon addition of 100 mM NaCl to the elution buffer, the two proteins in the mixture were separately eluted, indicating that the complex of Fd and SiR was formed mainly by electrostatic interaction (Fig. 5*B*). Titration of Fd in binding to SiR showed that the two proteins were bound with 1:1 stoichiometry (Fig. 5*C*).

The ability of each of the mutant Fds to bind to SiR was examined by gel filtration chromatography. As shown in Fig. 6*B,* E93Q and E30Q were unable to form the complex with SiR, and D66N/D67N and D61N were inferior to the wild type Fd. The other mutant Fds, D85N, D58N, D27N, and E71Q/E72Q, each showed complex-forming ability comparable to that of the wild type Fd. These results show a good correlation with those obtained by the enzymatic assay (Table II); E93Q, E30Q, D66N/D67N, and D61N with decreased binding ability showed 30–80% of the activity of the wild type Fd, whereas the other mutant Fds retained essentially wild type activity.

The same chromatographic assay of the mutant Fds was applied to examine complex formation with FNR. As shown in Fig. 6*A,* D66N/D67N could not form the complex with FNR, D61N, E71Q/E72Q, D27N, and E30Q each had less binding ability than the wild type Fd, and D85N, E93Q, D58N retained the same or similar ability. Although mutant Fds with lowered binding tended to show decreased electron transfer to FNR (Table II), there seems to be no good correlation between them as seen with SiR, because E71Q/E72Q, D27N, and E30Q, which displayed a diminished capacity for complex formation compared with the wild type Fd, still retained the full electron transfer activity. It is noteworthy that E93Q showed different characteristics compared with the other mutants. This mutant,

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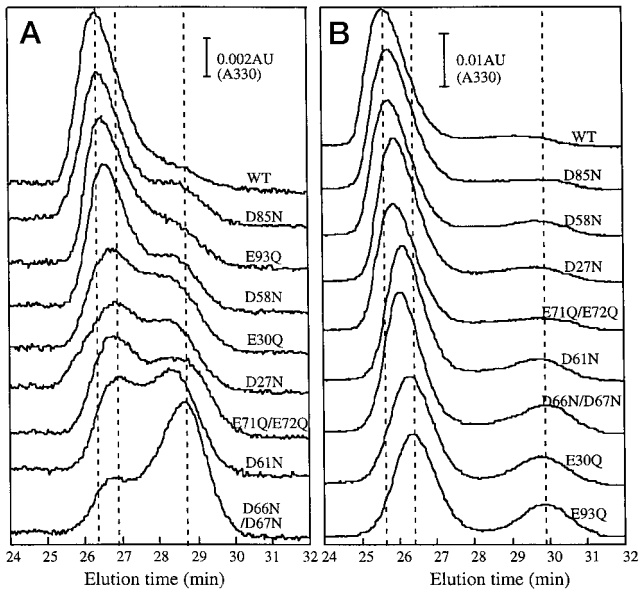

FIG. 6. **Co-chromatography of the mutated Fds and FNR (*A*) or SiR (*B*) on a gel filtration column.** *A,* a mixture of FNR and Fd in 1:1 stoichiometry was chromatographed on a Superdex 75 column. The complex of FNR and Fd, free FNR, and free Fd were eluted at 26.3, 26.8, and 28.7 min, respectively. *B,* a mixture of SiR and Fd in 1:1 stoichiometry was chromatographed on a Superdex 75 column. The complex of SiR and Fd, free SiR, and free Fd were eluted at 25.6, 26.4, and 29.8 min, respectively. The elution conditions are described under "Experimental Procedures." *WT,* wild type.

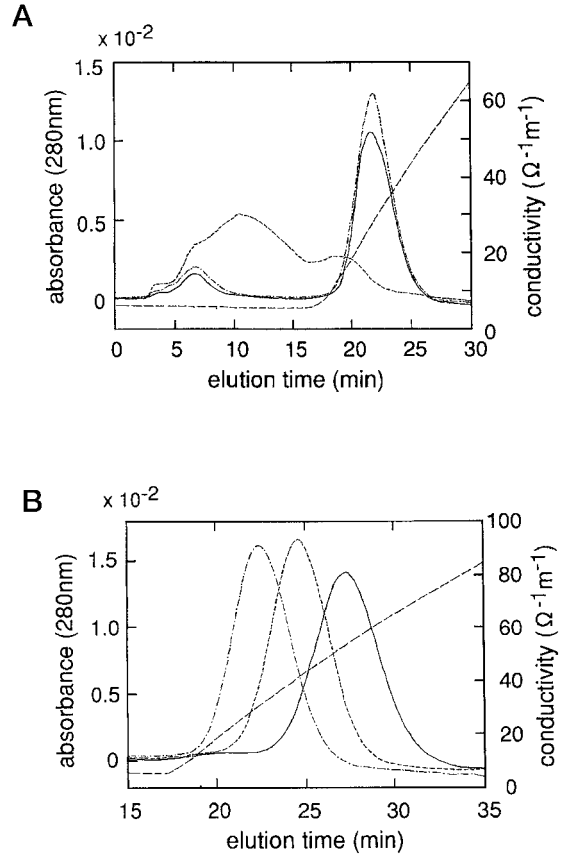

FIG. 7. **Analysis of the ability of FNR or SiR to bind to the mutated Fds by affinity chromatography.** *A,* FNR was loaded on three affinity columns with immobilized wild type Fd (——), D66N/D67N (- - - -), or E93Q (– – – –). The enzymes were eluted with a NaCl gradient from 0 to 400 mM in 50 mM Tris-HCl, pH 7.5. *B,* SiR was loaded on the three Fd affinity columns and eluted under the same conditions as in *A*. The conductivity of the eluate was monitored.

which was able to form a stable complex with FNR, but not with SiR, showed considerably lowered electron transfer ability toward both enzymes. This phenomenon seems to be due to the large positive shift in the redox potential.

Fd-Affinity Chromatography—Binding of FNR and SiR to the wild type Fd, D66N/D67N, and E93Q was further examined using Fd-immobilized Sepharose columns. As shown in Fig. 7*A,* FNR was not retained on the D66N/D67N column, whereas FNR became bound to the E93Q column and was eluted with a gradient of NaCl in a manner similar to that in the case of the wild type Fd column. SiR was eluted from the three Fd-affinity columns in the following order, E93Q, D66N/D67N, and wild type Fd (Fig. 7*B*). The differential binding of FNR and SiR to D66N/D67N and E93Q observed by Fd-affinity chromatography was in good agreement with the results obtained by gel filtration chromatography described in the above section.

DISCUSSION

Systematic site-directed mutagenesis of acidic residues located on the surface of maize Fd was successfully applied to map the regions involved in complex formation with FNR and SiR. Fig. 8 summarizes these results and shows the acidic residues contributing to formation of the complex with each of these enzymes.

Interaction of Fd with FNR—Regarding the sites involved in the interaction between Fd and FNR, the results of the present work are comparable to previous data obtained by chemical modification and computer modeling. By differential chemical modification of spinach Fd with 1-ethyl-3-[3-dimethyl-aminopropyl] carbodiimide/taurine in the presence and absence of spinach FNR, Asp-26, Glu-29, Glu-30, Asp-34, Asp-65, and Asp-66 (Asp-27, Glu-30, Thr-31, Asp-35, Asp-66, and Asp-67 in maize Fd) were protected from modification only when Fd was present as a complex with FNR (10). Computer modeling of the complex of *Spirulina* Fd and spinach FNR (3) suggested that Asp-28 and Glu-31 in the Fd (Asp-27 and Glu-30 in maize Fd) are close to Lys-300, Arg-301, Lys-304, and Lys-305 of the FNR, Asp-67 (Asp-66 in maize Fd) is close to Lys-33 and Lys-35, and Asp-62 (Asp-61 in maize Fd) is close to Lys-153. As shown in Fig. 8, most of these acidic residues in maize Fd are involved in the interaction with FNR. A cross-linking study of the complex of Fd and FNR indicated that the acidic residues at positions 92–94 in spinach Fd (93–95 in maize Fd) are the sites cross-linked to spinach FNR (17, 32). The present data, however, do not support the view that Glu-93 in maize Fd is the main site involved in binding to FNR. It seems that the cross-linked acidic residues may not necessarily contribute to electrostatic interaction in the Fd-FNR complex.

The degree of static interaction with FNR dose not show a clear correlation with the activity in electron transfer to FNR in the case of some Fd mutants. In the complex formation and electron transfer of Fd and FNR from spinach and *Anabaena*, transient kinetic measurements have suggested that the static interaction plays differing roles in controlling electron transfer between the two redox partners; one is stabilization of the Fd-FNR complex and the other is structural rearrangement within the transient complex, which optimizes the intracomplex electron transfer (20). The interaction measured by chromatographic techniques in this study do not necessarily reflect the ability of mutant Fds for such rearrangement of transient complex formation. Short range of forces, such as hydrophobic packing, van der Waals contact, and hydrogen bonding, on the top of the electrostatic force, also contribute to fine structural turning of the two redox partners. Although present data suggest some of acidic residues of maize Fd contribute mainly in an ensemble of loose interaction with FNR, but not in the fine

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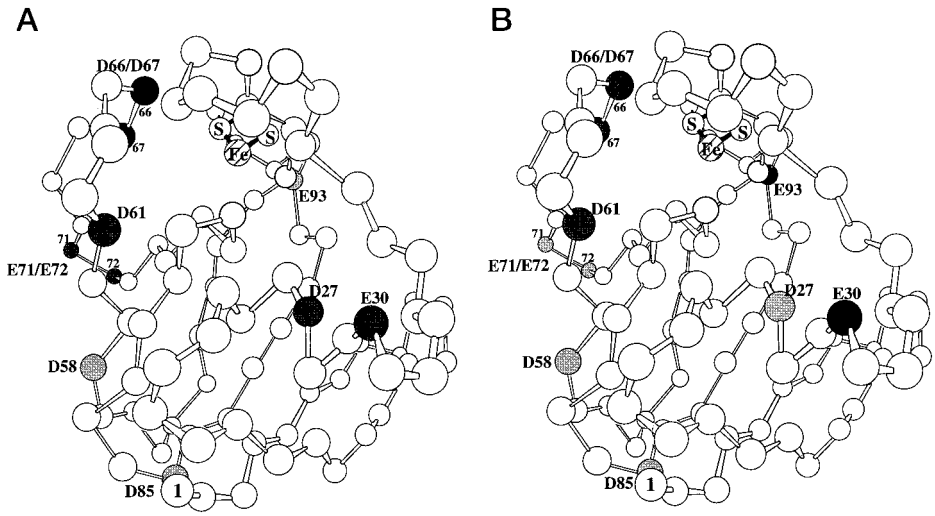

FIG. 8. **Comparison of the sites in Fd for electrostatic binding to FNR and SiR.** The sites of acidic residues in Fd are divided into three groups depending on the degree of contribution to the binding to FNR (*A*) and SiR (*B*): strong (*black*), medium (*gray*), and weak (*light gray*).

interaction productive for the efficient electron transfer, the precise explanations for this matter require further study.

However, the results of mutation at positions 66/67 were very straightforward; the negative charges in this region are crucial for both complex formation and electron transfer to FNR. Of these two acidic residues, Asp-66 seems to be physiologically important for the interaction with FNR. Among the maize Fd isoproteins, Fd I, Fd II, and Fd III, only Fd II has Asn at position 65 (corresponding to 66 in Fd III), and the K_m of FNR for Fd II was considerably higher than that for Fd I, whereas there was no substantial difference in V_{max} between the two Fds (33). In addition, the N65D mutant of Fd II showed increased activity, a level comparable to Fd I (33). The electrostatic interaction between Fd II and FNR was found to be weaker than that in the case of the other Fd isoproteins (data not shown). These data also support the view that the negative charge at this site is crucial for electron transfer between Fd and FNR.

Differential Sites in Fd for Interaction with Fd-dependent Enzymes—The present data demonstrate that the sites in Fd involved in binding to SiR are partly different from those for FNR; Glu-93 is the most important site for binding to SiR but not for binding to FNR. De Pascalis *et al.* (11) also proposed that there are different sites in Fd for binding to FNR and Fd-thioredoxin reductase as determined by chemical modification. Asp-34, Asp-65, Glu-92, Glu-93, and Glu-94 in spinach Fd (Asp-35, Asp-66, Glu-93, Gly-94, and Asp-95 in maize Fd) were shown to be close to the contact sites in the complex with spinach Fd-thioredoxin reductase, but not in the complex with spinach FNR. Comparing these findings with the present results, the sites involved in binding of Fd for Fd-thioredoxin reductase appear to be similar to those for SiR. The common sites in binding of Fd to FNR and SiR are mostly positioned around the [2Fe-2S] cluster. This suggests that although Fd may have a unique site for interaction with each Fd-dependent enzyme, the route of electron transfer from the [2Fe-2S] cluster of Fd to each of these enzymes is the same.

Regarding electron transfer to SiR, the static Fd-SiR interaction showed a good correlation with the kinetic activity in the case of most Fd mutants. This suggests that formation of the complex for electron transfer between Fd and SiR is largely dependent on electrostatic forces.

Role of Glu-93 in Determining the Redox Potential of the [2Fe-2S] Cluster—E93Q was found to be an inefficient electron carrier for transfer to both FNR and SiR, although this mutant interacts with FNR as well as the wild type. Recently, this glutamic acid residue at the same position was shown to be important for the reaction with a few Fd-dependent enzymes in the case of Fds from spinach (34), *Anabaena* (35), *Chlamydomonas* (36), and *Synechocystis* (37). E92Q, E92A, and E92K of spinach Fd and E94Q and E94K of *Anabaena* Fd showed a 60–90 mV positive shift in redox potential as observed in the case of the mutant E93Q of maize Fd. Thus, the change in redox potential appears to be the major factor responsible for the decrease in electron transfer ability. The negative charge at position 93 may be important to maintain the low redox potential of the [2Fe-2S] cluster, because the E94D mutant of *Anabaena* Fd showed a redox potential nearly equal to that of the wild type and because the mutant E94K with a positively changed side-chain showed a greater increase in redox potential than a mutant with a neutral side-chain (35).

According to the three-dimensional structure of the *Anabaena* Fd (38), the side-chain of Glu-94 was proposed to play a role in stabilizing the [2Fe-2S] cluster binding loop of the polypeptide backbone through a hydrogen bond with the side-chain of Ser-47 (Ser-46 in maize Fd). The crystal structure of mutant E94K of *Anabaena* Fd, which lacks the hydrogen bond, indicates that this mutation caused only a minor perturbation in the vicinity around the [2Fe-2S] center (39). We are also currently studying the x-ray structure of S46G mutant of maize Fd, which shows about 180 mV positive shift (31), and the preliminary data suggest that only a local configuration of the [2Fe-2S] cluster binding loop is perturbed in this mutant.[2] These combined data suggested that at least two factors, a small change in the conformation around the [2Fe-2S] cluster and the introduction of neutral and positively charged side-chains at position 93, which may result in stabilization of the [2Fe-2S] cluster with an overall negative charge, should induce significant redox changes.

In conclusion, we have demonstrated that the ability of Fd to transfer electrons to two Fd-dependent enzymes, FNR and SiR, is decreased by lowering the ability of Fd to form a stable complex with each of the enzymes by mutagenesis of specific acidic residues. We have also found that Fd has electrostatic interaction sites both common and unique to each enzyme. This specific Fd-enzyme interaction site might be an important factor in regulating the distribution of electrons among several Fd-dependent enzymes in various metabolic pathways of chloroplasts.

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