NMR Study of the Electron Transfer Complex of Plant Ferredoxin and Sulfite Reductase

MAPPING THE INTERACTION SITES OF FERREDOXIN

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Plant ferredoxin serves as the physiological electron donor for sulfite reductase, which catalyzes the reduction of sulfite to sulfide. Ferredoxin and sulfite reductase form an electrostatically stabilized 1:1 complex for the intermolecular electron transfer. The protein-protein interaction between these proteins from maize leaves was analyzed by nuclear magnetic resonance spectroscopy. Chemical shift perturbation and cross-saturation experiments successfully mapped the location of two major interaction sites of ferredoxin: region 1 including Glu-29, Glu-30, and Asp-34 and region 2 including Glu-92, Glu-93, and Glu-94. The importance of these two acidic patches for interaction with sulfite reductase was confirmed by site-specific mutation of acidic ferredoxin residues in regions 1 and 2, separately and in combination, by which the ability of mutant ferredoxins to transfer electrons and bind to sulfite reductase was additively lowered. Taken together, this study gives a clear illustration of the molecular interaction between ferredoxin and sulfite reductase. We also present data showing that this interaction surface of ferredoxin significantly differs from that when ferredoxin-NADP+ reductase is the interaction partner.

Plant sulfite reductase (SiR) (EC 1.8.7.1) plays an essential role in the reductive assimilation of sulfate by plants, algae, and cyanobacteria by catalyzing the six-electron reduction of sulfite to sulfide (1). The enzyme is a soluble, monomeric protein with a molecular mass of ~65 kDa and contains a single siroheme and a single [4Fe-4S] cluster as prosthetic groups (2, 3). Plant-type ferredoxin (Fd) is a small (11 kDa), one-electron carrier protein with a single [2Fe-2S] cluster and serves as the physiological electron donor for SiR (4). The midpoint redox potential (Em) for the [4Fe-4S] cluster in maize SiR are –400 and –285 mV, respectively (5). Therefore, electron flow to the enzyme from the reduced Fd, which has an Em value ~ –400 mV, is thermodynamically favorable.

A transient electron transfer complex is formed between Fd and SiR with 1:1 stoichiometry to facilitate efficient intermolecular electron transfer (6, 7). Other Fd-dependent enzymes of varying molecular size, primary structure, and prosthetic group composition, such as Fd-NADP+ reductase (FNR), nitrite reductase, glutamate synthase, and Fd-thioredoxin reductase, also form productive electron transfer complexes with Fd. Several lines of evidence obtained from chemical modification experiments, cross-linking experiments, and mutagenesis experiments have indicated that the complexes between Fd and these Fd-dependent enzymes are mainly stabilized by electrostatic forces through the negative charges of Fd and positive charges of each enzyme (8). An example can be seen in the recently determined crystal structure of the complex between maize leaf Fd and FNR (9). The redox centers of Fd and FNR are in close proximity in the complex to allow fast electron transfer between them. The intermolecular contact site near the redox centers is hydrophobic, and five intermolecular salt bridges are formed around this hydrophobic region to determine the orientation of the two proteins (9). However, no crystal structure of the complex between Fd and any other Fd-dependent enzyme, including SiR, has yet been reported.

A series of site-directed mutagenesis experiments on maize Fd demonstrated that certain acidic residues were crucial for interaction and electron transfer with SiR, as is the case for interaction with FNR (7). A recent study on chemical modifications of lysine and arginine residues of maize SiR showed that some basic residues are also involved in SiR binding to Fd (10).

Two-dimensional NMR is a useful technique to study such transient protein-protein interactions of complexes between electron carrier proteins and their partner enzymes (11, 12, 13). This technique was successfully applied to the study of the electron transfer complex between Fd and FNR (9). Using Fd enriched in 15N stable isotope, a heteronuclear single quantum correlation (HSQC) experiment correlated the resonance of the amide15N and1H nuclei, and any other Fd-dependent enzyme, including SiR, has yet been reported.

In this study we have further applied this kind of NMR analysis to elucidate the details of the contact area on Fd in the transient complex formed with SiR. In addition, a new measurement called NMR cross-saturation (14), using 15N- and 2H-labeled Fd, enabled us to obtain information about the interface. The importance of acidic residues in the contact sites was further demonstrated through mutagenesis experiments. By comparing the detected contact areas of Fd on interaction with SiR and FNR, the structural characteristics of Fd that enable it to form complexes with two different enzymes will be discussed.
EXPERIMENTAL PROCEDURES

Preparation—Sample Fd isoprotein from maize leaf (Fd I) (15) was used in this study. Site-specific mutants of Fd, E29Q/E30Q/D34N, E92Q/E93Q, and E29Q/E30Q/D34N/E92Q/E93Q, were prepared with the QuikChange site-directed mutagenesis kit (Stratagene). The synthetic oligonucleotides used for the mutagenesis were CCTGGACCGGGCCCAGCAGGACGGCATCAACCTGCCCTACTCCTGC and GCAGGAGTAGGGCAGGTTGATGCCGTCCTGCTGGTCCTCCAGG for E29Q/E30Q/D34N, and CGTCATCGAGACGCACAGGCAGCAGGAGCTCACCGGCGC and GCGCCGGTGAGCTCTGCTTGTGCGTCTCGATGACG for E92Q/E93Q, where the underlined bases denote changes from the wild-type sequence. The mutation sites and the sequence integrity of the entire coding regions of the Fds were confirmed by DNA sequencing.

Wild-type and mutant molecules of maize Fds were expressed in Escherichia coli strain JM109 cells and purified as described previously (16). 15N-labeled Fds were obtained by culturing the bacterial cells in an M9 minimum medium containing 15NH4Cl as the sole nitrogen source. 2H-, 15N-labeled Fd was obtained by the same procedure, except that medium was prepared with 99% D2O containing [2H6]-D-glucose and 15NH4Cl. Maize SiR was expressed in E. coli and purified as described previously (3).

CD Spectroscopy—CD spectra of WT and mutant Fds were analyzed using a JASCO J720WI spectrometer. Fds were dissolved in a 50-mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl at the final protein concentrations of 91 and 9.1 M for the measurements in the visible and UV regions, respectively.

NMR Spectroscopy—For chemical shift perturbation experiments, 2H, 15N-labeled or 15N-labeled Fd proteins were dissolved at a concentration of 0.1–0.2 mM in a 30-mM potassium phosphate buffer (pH 7.0) containing 25 mM NaCl and 10% D2O. 1H–15N transverse-relaxation optimized spectroscopy (TROSY) HSQC spectra of 2H, 15N-labeled Fd samples mixed with increasing amounts of non-labeled SiR at molar ratios of 0, 0.25, 0.5, 0.75, 1.0, 1.33, 1.67, 2.0, and 3.0 were recorded at 298 K on a Bruker DRX800 spectrometer equipped with a cryogenic probe, operated at the 1H base resonance frequency of 800.13 MHz. 1H–15N HSQC spectra of the 15N-labeled wild-type Fd samples mixed with increasing amounts of non-labeled SiR at molar ratios up to 1.0 were recorded at 298 K on a Bruker AV400M spectrometer, operated at the 1H base resonance frequency of 400.13 MHz. 1H–15N HSQC spectra of the 15N-labeled E29Q/E30Q/D34N/E92Q/E93Q mutant mixed with increasing amounts of non-labeled SiR at molar ratios of 0, 0.75, and 2.0 were recorded at 298 K on the Bruker DRX800 spectrometer with a cryogenic probe. All the spectra contained WATARGATE just before the direct detection period (17). Weighted averaged 1H and 15N chemical shift changes were calculated with the equation $\Delta\delta_{\text{av}} = \left(\Delta\delta_{1HN}^2 + (0.17\Delta\delta_{15N})^2\right)^{1/2}$.

Cross-saturation experiments (14, 18) were employed at 298 K on a Bruker DRX600 spectrometer, operated at the 1H base resonance frequency of 600.13 MHz, or on the Bruker DRX800 spectrometer with a cryogenic probe. Fd, labeled uniformly with the 15N and 2H isotopes, was dissolved at a concentration of 0.5–1 mM in a 30-mM potassium phosphate buffer (pH 6.8) containing 25 mM NaCl and 90% D2O. The HSQC part of the pulse sequence was constructed by a normal INEPT and reverse INEPT type (17, 18). The radio frequency wave for the saturation was applied for 2.0 s just before the HSQC part by 200 times of sequential repeats of an adiabatic pulse with an amplitude modulation of a hyperbolic secant, a frequency sweep range of 1000 Hz, a duration of 10 ms, a maximum amplitude of 200 Hz, and an excitation center at 1.5 or 0.9 ppm by the phase modulation. The simulation confirmed that the two kinds of pulse excited only aliphatic and methyl resonance regions and had no effect of perturbation on the water resonance. All the spectra were processed with NMRPipe software (19) and analyzed with Sparky software developed by T. D. Goddard and D. G. Kneller at University of California, San Francisco.

FIGURE 1. 1H–15N TROSY HSQC spectra of 2H, 15N-labeled Fd. Overlay of the HSQC spectra in the absence (green) and presence (red) of non-labeled SiR at a molar ratio ([SiR]/[Fd]) of 2.0. The assignments to the backbone amide groups are indicated in black for cross-peaks with no or little chemical shift change and in blue for those with a significant change. See “Experimental Procedures” for detail.
Affinity Chromatography—10 mg of SiR was immobilized on 2 g of CNBr-activated Sepharose 4B (Amersham Biosciences) according to the method recommended by the supplier. A small column packed with 2 ml of SiR-immobilized resin was mounted onto a chromatography system (AKTA prime; Amersham Biosciences). After equilibrating the column with 50 mM Tris-HCl, pH 7.5, 100 µl of 100 µM wild-type Fd or mutant Fd was loaded onto the column and eluted with a linear gradient of NaCl from 0 to 450 mM at a flow rate of 0.2 ml/min. Elution of Fd was monitored by the absorbance at 430 nm (A_{430}), and the conductivity of the eluate was correspondingly monitored.

Enzyme Assay—Activity of SiR was assayed with a reconstituted electron transfer system from NADPH to sulfite using FNR as described previously (4). The reaction mixture contained Fd (0–40 µM), FNR (100 nM), SiR (800 nM), and sulfite (0.5 mM) in 30-mM potassium phosphate buffer, pH 7.0 and 25 mM NaCl. The reaction was initiated by adding NADPH (0.2 mM), and the oxidation of NADPH was monitored by the decrease at A_{340}.

RESULTS

Chemical Shift Perturbation Experiment—A series of {H,}^{15}N TROSY HSQC spectra of {H,}^{15}N-labeled Fd was measured as a function of the molar ratios of non-labeled SiR to Fd. As shown in Fig. 1, the amide resonance of most Fd residues was well observed except for those in three regions: Ser-38 to Ala-48, Ser-62, and Thr-76 to His-78, whose signals were hardly detected due to paramagnetic relaxation enhancement caused by the [2Fe-2S] cluster of Fd. A general broadening of the resonance signals that should have occurred upon addition of SiR was minimized by the TROSY effect, and concomitant chemical shift changes were well observed only for certain residues. Titration curves of the chemical shift changes upon addition of SiR are shown for representative residues perturbed to varying extents (Fig. 2); all residues exhibited an almost saturated state in their chemical shift change at the molar ratio of ~1.5. This type of resonance shift change between the
extreme shifts in the free and complex forms indicated that the two states underwent fast exchange on the NMR time scale. An average $K_d$ value was calculated as $12.9 \pm 3.2 \mu M$ from the titration curves of 20 residues, showing significant chemical shift changes.

For each amide group of Fd, the changes in the $^1$H and $^{15}$N chemical shifts were combined according to the equation described under “Experimental Procedures,” and the resulting values are shown in Fig. 3a. The Fd residues that exhibited significant chemical shift perturbations ($\geq 0.08$ ppm and $0.06-0.08$ ppm) are graphically visualized on the three-dimensional structure in two color codes (Fig. 3b). Complex formation with SiR significantly influenced the resonance of Glu-13, Ile-24, Leu-25, Ala-28, Glu-29, Glu-30, Gly-32, Asp-34, Leu-35, Tyr-37, Gln-61, Tyr-63, Leu-64, His-90, Lys-91, Glu-92, Glu-93, Glu-94, and Ala-98. These residues are distributed in areas surrounding the [2Fe-2S] cluster; most are located on the surface of the Fd molecule, although some of the moderately influenced residues such as Ile-24 and Leu-25 are buried inside the molecule. We assume that most of the amide resonance changes are due to protein-protein interaction of Fd and SiR.

A series of conventional $^1$H-$^{15}$N HSQC spectra, instead of the TROSY version, $^{15}$N-labeled Fd was also measured in the presence of SiR at the molar ratios of 0.25, 0.5, 0.75, and 1.0. Under the highest molar ratio of 1.0, the resonance derived from 15% of Fd amide groups was broadened to a large extent, most probably because of the large molecular size of the complex, and unambiguous assignment of the peak positions was almost impossible. Although the degrees of the broadening effects observed in the conventional and TROSY $^{15}$N-HSQC spectra were outstandingly different, we confirmed that the patterns in the chemical shift perturbation, such as the magnitude and direction of each shift, were the same at least in the respective spectra at a molar ratio of 0.75 (supplemental Fig. S1).

Cross-saturation Transfer Experiment—To further investigate the interaction of Fd with SiR, a cross-saturation experiment was performed. Upon complex formation with SiR, saturation of the proton magnetization in the whole SiR can be transferred to the interaction interface of Fd, and thereby the intensity of cross-peaks derived from the amide protons in interaction sites is decreased. For this experiment, Fd was labeled uniformly with $^2$H ($\geq 97\%$) and $^{15}$N to avoid the magnetization of any aliphatic and methyl proton of Fd from being saturated directly by the radiofrequency pulses. This labeled Fd was dissolved in a buffer of 10$\%$ $^1$H$_2$O and 90$\%$ $^2$H$_2$O to decrease the saturation transfer through $^1$H$_2$O and spatially crowded amide protons. We set the center of the irradiation at 1.5 ppm such that the irradiation pulses covered the aliphatic proton resonance region but at the same time did not perturb the water magnetization. Typical $^1$H-$^{15}$N TROSY HSQC spectra of Fd with and without the irradiation in the presence of SiR at the molar ratio of 0.5 are shown in Fig. 4a. The intensity of cross-peak of Gly-32 predominately decreased in the saturation condition. The intensity ratios of the cross-peaks for all the observed Fd residues in the presence and absence of SiR are shown in Fig. 4b. A selective saturation effect was observed in a region containing residues Glu-30, Asp-31, Gly-32, Ile-33,
TABLE 1

Kinetic parameters of SiR for wild-type and mutant Fds in NADPH-sulfite reduction

These data were extracted from Fd saturation curves, and 𝑘_{𝑤𝑡} and 𝑘_{𝑚𝑡} were determined from a double-reciprocal plot. The values are mean ± S.D. of three independent measurements.

|          | 𝑘_{𝑤𝑡} | 𝑘_{𝑚𝑡} | 𝑘_{𝑖𝑛} | 𝑘_{𝑤𝑡}/𝐾_{𝑖𝑛} |
|----------|--------|--------|--------|---------------|
| WT       | 4.0 ± 0.2 | 3.65 ± 0.02 | 0.91 |
| DM       | 6.2 ± 1.1 | 2.70 ± 0.10 | 0.44 |
| TM       | 9.5 ± 2.2 | 2.66 ± 0.24 | 0.28 |
| QM       | 14.7 ± 2.3 | 2.10 ± 0.05 | 0.14 |

a WT, wild-type Fd.

b DM, E92Q/E93Q.

c TM, E29Q/E30Q/D34N.

d QM, E92Q/E93Q/E29Q/E30Q/D34N Fds.

and Asp-34 that showed significantly reduced ratios in comparison with other residues. When these results were compared with the chemical shift mapping data (Fig. 3), these residues were located at or near the regions where a large chemical shift perturbation was observed.

We also examined the cross-saturation effect when the region for methyl protons was irradiated by shifting the excitation center to 0.9 ppm as shown in supplemental Fig. S2. A strong saturation effect was observed again in the residues from Gly-32 to Asp-34, confirming the above result obtained at the excitation center at 1.5 ppm. Some of the residues with methyl groups exhibited low intensity ratios even in the absence of SiR. This phenomenon was most probably caused by a small amount of H being mixed into the methyl groups of Fd side chains.

Effect of Mutation at the Interaction Interface of Fd—NMR experiments indicated that the two acidic patches on the surface of Fd are involved in interaction with SiR, one region containing Glu-29, Glu-30, and Asp-34 and the other containing Glu-92, Glu-93, and Glu-94 (Fig. 3b). To confirm the importance of these regions to protein-protein interaction, the acidic residues contained in each or both of these regions were substituted to the corresponding amide form and their electron transfer ability to SiR was examined by kinetic analysis. Three of the mutants generated, E92Q/E93Q (DM), E29Q/E30Q/D34N (TM), and E29Q/E30Q/D34N/E92Q/E93Q (QM) Fds, showed a significantly lower electron transfer ability compared with WT Fd as shown by kinetic parameters in Table 1. QM Fd retained the least ability among the mutant Fds. Physical binding of these mutant Fds to SiR was further confirmed by the above result obtained at the excitation center at 1.5 ppm.

Comparison of the Interaction Interface of Fd with SiR and FNR—The atomic structure of the electron transfer complex of plant Fd and FNR was determined by x-ray crystallographic analysis, and details of their protein-protein interaction are now known (9). The same complex was also analyzed with NMR chemical shift perturbation technique, and the Fd regions that interacted with FNR in solution were in good agreement with those determined by the crystallographic data (9).

We also examined the cross-saturation effect when the resonance of the 15N-labeled Fd in the presence of FNR or SiR as a side by side experiment. As shown in Fig. 7a there are significant differences in the chemical shift change patterns between the two complexes. In the complex with FNR, the acidic patch consisting of Asp-65 and Asp-66 along with Glu-61, Leu-64, and Gln-68, which showed remarkable chemical shift perturbations, is the major interaction site on Fd with FNR (Fig. 7b). In addition to this acidic patch, the resonance of the C-terminal tail from Leu-95 to Ala-98 of Fd also strongly shifted upon complex with FNR. In the previous report Asp-60 was also strongly shifted (9), but the present data in Fig. 7a show no such significant perturbation. We observed that perturbation of this residue sharply decreased with increasing salt concentration and confirmed that under previous low salt conditions (in the presence of 10 mM NaCl) a large perturbation was observed (data not shown). Mapping of the interaction site on Fd with FNR (Fig. 7b) significantly differs when the interaction partner is SiR (Fig. 7c), indicative of two different interaction ways of Fd toward the respective enzymes, although the [2Fe-2S] cluster seems to be located at the interface of Fd and the partner enzyme in both cases.

DISCUSSION

We observed significant chemical shift perturbation of the 1H-15N HSQC spectra of Fd upon interaction with SiR. Biochemical data show that Fd and SiR form a complex of 1:1 stoichiometry and that the affinity of SiR for Fd in the reaction of Fd-dependent sulfite reduction is high, as demonstrated by the 𝑘_{𝑖𝑛} value of ~5 μM (3, 7, 10). In the present study, the physical binding constant between SiR and Fd has been determined to be 13 μM from the titration results of chemical shift changes in the TROSY HSQC spectra. This value in the μM range agrees well with the 𝑘_{𝑖𝑛} value determined from the SiR activity assay using Fd as an electron donor. This fact suggests that the complex of Fd and SiR observed by our NMR measurements corresponds to the one that is kinetically competent for intermolecular electron transfer.
This NMR study gives a clear picture of the molecular interaction mode of Fd with SiR. The Fd residues with the most significant alterations in NMR spectra are Ala-28 to Tyr-37, Gln-61 to Leu-64, and His-90 to Glu-94, and these three regions are located around the [2Fe-2S] cluster on the three-dimensional structure of Fd (Fig. 3). Chemical shift changes could be interpreted as arising either from direct protein-protein interactions or from secondary effects, such as conformational change beyond the contact interface and/or alteration in electronic shielding upon complex formation. The cross-saturation experiment concluded that the region around Gly-32 is most probably located at the intermolecular interface and plays a role in the interaction between Fd and SiR. The importance of this region was also confirmed by further NMR measurements and other biochemical analyses of the complex formation by using mutant Fds. Cross-saturation effect was not so clearly detected for the two regions around Gln-61 to Leu-64 and His-90 to Glu-94, where large chemical shift changes were, however, observed.
Electron Transfer Complex of Ferredoxin and SiR

As the saturation transfer efficiency depends mainly on the distance between the donor and acceptor sites of saturated magnetization, these two Fd regions may not be so close to the SiR surface as the region around Ala-28 to Tyr-37. Nevertheless, the possibility that these two regions are far from the interaction site does not seem to be high, because the mutation of Glu-92 and Glu-93 exhibited a significant effect on the binding of Fd to SiR. Therefore, we believe that our mapping of the chemical shift changes well reflects features of the protein-protein interaction between Fd and SiR.

The results of gel filtration chromatography (7) and spectral perturbation experiments (10) showed that Fd and SiR form a complex of high affinity at a lower ionic strength but such molecular interaction does not occur at a higher ionic strength, indicative of the major contribution of electrostatic force to stabilizing the complex. Previous experiments, using site-specific mutants of root-type Fd isofrom from maize, demonstrated that negatively charged residues of Fd participate in this electrostatic interaction (7). Of the three regions of Fd that we have proposed as being involved in interaction with SiR, two regions are rich in acidic residues forming a negatively charged molecular surface; one contains Glu-29, Glu-30, and Asp-34 and the other Glu-92, Glu-93, and Glu-94. The results of experiments on root Fd (7) are consistent with the conclusions obtained from leaf Fd in this study, suggesting that both types of Fd interact with SiR in a similar way. The acidic patches seem to be cooperatively involved in electrostatic interaction with SiR, because the simultaneous mutation of both patches resulted in more remarkable loss of ability in interaction with SiR than the separate mutations of each patch (Fig. 5). Thus, one would expect that at least two positively charged surfaces on SiR operate as the counterparts of the Fd acidic patches when the complex is formed. This was experimentally suggested by chemical modification of lysine and arginine residues of SiR that caused an inhibitory effect on Fd-dependent activity of SiR, but not on redox dye-dependent activity (10).

The two acidic patches of Fd are conserved among Fds from various species. By site-specific mutational study of several Fds, it has been reported that the two acidic patches are involved in the interaction with Fd-thioredoxin reductase in spinach (20) and Chlamydomonas (21) and nitrite reductase in Chlamydomonas (22). These data suggest a general importance of the common acidic patches for interaction with different Fd-dependent enzymes. However, this is not the case for FNR. When the profile of chemical shift changes of Fd in complex with FNR is compared with data of the complex with SiR (Fig. 7a), the regions of Fd forming the two acidic patches (Ala-28 to Leu-35 and His-90 to Glu-94) show no remarkable chemical shift changes, whereas a separate acidic region around Glu-65 and Glu-66 that is also evolutionally conserved showed the largest chemical shift change in the complex with FNR. In fact, Glu-65 and Glu-66 of Fd form strong salt bridges with Lys-91 and Lys-88 of FNR, respectively, as shown in the crystal structure of the Fd-FNR complex (9). This suggests that the binding sites of Fd vary appreciably between FNR and SiR as visualized in Fig. 7, b and c.

The NMR signals for residues Ser-38 to Ala-49, Ser-62, and Thr-76 to His-78 are not observed due to paramagnetic relaxation enhancement of the iron atoms of the [2Fe-2S] cluster, and it is difficult to draw the features of the overall topology of Fd for interaction with partner enzymes from NMR data only. In the crystal structure of the complex between Fd and FNR, NMR undetectable residues produce a hydrophobic environment at the interface of the two proteins, which allows a direct electron transfer between the [2Fe-2S] cluster of Fd and flavin adenine dinucleotide (FAD) of FNR over a distance of 6 Å (9). The area surrounding this hydrophobic region, which contributes to fine adjustment of the topology of the two proteins, shows large chemical shift changes (Fig. 7b). In the case of the complex of Fd and SiR, almost all residues showing large chemical shift changes are also distributed in the side of the [2Fe-2S] cluster; therefore, it is reasonable to predict that the complex of Fd and SiR forms in a way that the [2Fe-2S] cluster side is facing to the electron acceptance side of SiR, although the details of intermolecular electron transfer are not predictable without information of the three-dimensional structure of the Fd-SiR complex. Fd is an electron carrier that distributes reducing power to various redox enzymes in chloroplasts and non-photosynthetic plastids. To understand this multifunctionality of Fd, early investigations into the molecular evolution of Fd (23) and interactions between Fd and Fd-dependent enzymes (24) postulated that the conserved acidic residues, which are distributed across several areas on the Fd surface, are crucial for specific interaction with various Fd-dependent enzymes. However, it has not been well understood whether Fd interacts with partner enzymes in a similar way or whether Fd has binding domains specific for each enzyme. Although no crystal structure of the complex of Fd and Fd-dependent enzymes other than FNR is available yet, this study can conclude that at least two enzymes such as SiR and FNR utilize a different combination of the acidic regions of Fd to form a complex suitable for intermolecular electron transfer. We are now investigating the above questions in more detail by applying this NMR technique to other Fd-dependent enzymes.

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