NMR-detected hydrogen/deuterium (H/D) exchange of amide protons is a powerful way for investigating the residue-based conformational stability and dynamics of proteins in solution. Maize ferredoxin-NADP⁺ reductase (FNR) is a relatively large protein with 314 amino acid residues, consisting of flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADP⁺)-binding domains. To address the structural stability and dynamics of FNR, H/D exchange of amide protons was performed using heteronuclear NMR at pD values 8.0 and 6.0, physiologically relevant conditions mimicking inside of chloroplasts. At both pD values, the exchange rate varied widely depending on the residues. The profiles of protected residues revealed that the highly protected regions matched well with the hydrophobic cores suggested from the crystal structure, and that the NADP⁺-binding domain can be divided into two subdomains. The global stability of FNR obtained by H/D exchange with NMR was higher than that by chemical denaturation, indicating that H/D exchange is especially useful for analyzing the residue-based conformational stability of large proteins, for which global unfolding is mostly irreversible. Interestingly, more dynamic conformation of the C-terminal subdomain of the NADP⁺-binding domain at pD 8.0, the daytime pH in chloroplasts, than at pD 6.0 is likely to be involved in the increased binding of NADP⁺ for elevating the activity of FNR. In light of photosynthesis, the present study provides the first structure-based relationship of dynamics with function for the FNR-type family in solution.

Cores and pH-dependent Dynamics of Ferredoxin-NADP⁺ Reductase Revealed by Hydrogen/Deuterium Exchange*§

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Protein conformations as shown by x-ray crystallography or nuclear magnetic resonance spectroscopy are virtually dynamic entities over various time ranges in solution (1, 2). Clarifying such conformational motions at the level of the atom or residue is essential for understanding the structural stabilities of proteins and the relationships between protein structures and functions (3–5). The hydrogen/deuterium (H/D) exchange of amide protons in backbones has become an important way to address the motions of a protein from small or relatively large scale motions involved in biological functions such as binding of a substrate or releasing a product to much more large scale motions like a global unfolding process (4, 6). Among several approaches, the H/D exchange combined with heteronuclear NMR spectroscopy is the most convenient and powerful way because it can provide residue-specific information for most residues (7). For many globular proteins, this approach has identified protected cores, which are often composed of secondary structures buried inside the molecules and the cooperative interactions with partner molecules (4, 8), leading to allostery (9). Detailed analyses of exchanges in the native state of cytochrome c in the presence of various concentrations of denaturants suggested a pathway to unfolding, in which groups of secondary structural elements (i.e. foldons) are unfolded sequentially through distinct folding intermediates (10).

Although the impact of the H/D exchange method combined with NMR analyses is obvious, its application has been limited to proteins for which the amide resonances were already assigned. Therefore, the measurements have been performed with relatively small proteins, mostly less than 20,000 in a molecular weight (11). The largest proteins examined so far are bovine β-lactoglobulin (162 residues) (7), T4 lysozyme (164 residues) (12), and scFv (252 residues but with the same two domains) (13). Moreover, because the unfolding of proteins with a molecular weight greater than 20,000 is usually irreversible, a quantitative kinetic analysis of H/D exchange has not been established for large proteins.

Ferredoxin-NADP⁺ reductase (FNR, 1.18.1.2) from maize leaf is a ubiquitous protein consisting of 314 amino acid residues. It catalyzes the transfer of electrons during photosynthesis: FNR receives two electrons from ferredoxin, a one-electron carrier protein, and transfers them to NADP⁺ to produce NADPH (14). The activity and affinities for substrates of FNR decreased in response to a pH change from about 8 to 6 in chloroplasts (15). The three-dimensional structure of maize FNR composed of the well defined FAD- and NADP⁺-binding domains has been solved by x-ray crystallography (16). The...
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FAD-binding domain (residues 1 to 153) is made up of a six-stranded antiparallel β-barrel the bottom of which is capped by an α-helix and a long loop. The NADP⁺-binding domain (residues 154 to 314), a variant of the typical dinucleotide-binding fold (17, 18), consists of a central five-stranded parallel β-sheet surrounded by six α-helices. The FAD cofactor is tightly bound to the FAD-binding domain with its isoalloxazine ring sandwiched between the two domains, whereas the substrate NADP⁺ binds to the NADP⁺-binding domain. The binding of FAD is essential for maintaining the native structure, so that apo-FNR, lacking FAD, is assumed to have an intermediate conformation with a largely unfolded FAD-binding domain (19).

We have been studying the conformation and function of maize leaf FNR through heteronuclear NMR analyses with the recently achieved assignment for more than 95% of its amide protons (20). In the present study, taking advantage of the NMR assignment, we investigated the conformational stability of FNR using the H/D exchange of the amide protons. The results revealed the three protected core regions of FNR in which the NADP⁺-binding domain can be divided into two subdomains with distinct differences in their motions. Moreover, the C-terminal subdomain exhibited a pH-dependent change of motions, implying a potential role in adjusting binding affinity for NADP⁺ leading to effective functional control for photosynthesis in the daytime. Comparison of the apparent free energy change of unfolding (ΔGobs/α) estimated for each residue on the basis of H/D exchange with the global free energy change of unfolding (ΔGobs) estimated from urea-induced unfolding suggests that H/D exchange provides a more reliable estimate of the stability of the core regions.

EXPERIMENTAL PROCEDURES

Sample Preparation—The Escherichia coli strain TG1 cells transformed with the plasmids containing the DNA of FNR were inoculated in 50 ml of a Luria-Bertani (LB) medium and the bacteria were further incubated at 37 °C. The expression of the proteins was initiated by adding isopropyl 1-thio-D-galactopyranoside when the cell density reached an absorbance of 0.65 at 600 nm. After additional incubation for 12 h, the cells were collected by centrifugation and disrupted by ultrasonication and the supernatant was applied to an anion-exchange column, DE52 (Whatman). The flow-through fraction was fractionated with ammonium sulfate at 40–70% saturation. The resulting precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 40% ammonium sulfate, and the solution was loaded onto a gel-filtration column of Sephadex G-25 (Amersham Biosciences), pre-equilibrated with a 50 mM Tris-HCl buffer (pH 7.5). Unless otherwise noted, all chromatographic columns were equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The FNR fraction was further separated by anion exchange chromatography with DEAE-Toyopearl (Tosoh, Japan). FNR was eluted by applying a linear gradient of the NaCl concentration from 0 to 0.5 M in the same buffer, monitored by measuring absorbance at 280 nm in the AKTA system (Amersham Biosciences). In the last purification step, an affinity chromatography of a Fd matrix was used as described previously (19, 20). The purity of FNR was confirmed by SDS-polyacrylamide gel electrophoresis. The protein concentration was determined by absorption using extinction coefficients of 10,000 M⁻¹ cm⁻¹ at 460 nm and 47,800 M⁻¹ cm⁻¹ at 280 nm.

To obtain a uniformly 15N-labeled FNR for NMR measurements, a colony of the transformed cells was grown in 50 ml of LB medium containing 0.14 mM ampicillin at 37 °C for 6 h, and then 10 ml of the medium was inoculated with 1.5 liters of an M9 minimum medium containing 1.24 g/liters of (15NH₄)₂SO₄ as a sole nitrogen source. The procedure used to purify 15N-labeled FNR was identical to that used for non-labeled FNR.

CD and Fluorescence Measurements—CD measurements were carried out in a J-720WI spectropolarimeter (Jasco, Japan) using a cell with a 1-mm light path at a protein concentration of 4 μM. The results were expressed as the mean residue ellipticity, [θ], defined as [θ] = 100 θobs/l c, where θobs is the observed ellipticity in degrees, c is the concentration in residue moles/liter, and l is the length of the light path in cm. The temperature was controlled by a Jasco PTC-348WI peltier system. Fluorescence emission spectra were recorded at 25 and 40 °C in a 25 mM sodium phosphate buffer (pH 8.0), using an F-4500 fluorometer (Hitachi, Japan) and at 40 °C in the same buffer (pH 6.0) in the presence and absence of 50 mM NaClO₄. For the fluorescence spectrum of FAD, it was excited at a wavelength of 453 nm. The protein concentration was set at 1 μM, and the temperature was maintained by a thermostatically controlled water bath. Equilibrium denaturation experiments were done as follows. Protein solutions containing various concentrations of urea were preincubated for at least 24 h at room temperature prior to the experiments. The transitions were followed by measuring the far UV CD and fluorescence intensity of FAD. For CD measurements, the change in ellipticity at 222 nm was recorded. Relative fluorescence intensity was recorded at 530 nm with an excitation wavelength of 453 nm.

The resulting transition curves were analyzed by non-linear least squares curve fitting assuming a two-state transition (21) as shown in the following equation.

\[
[\text{Signal}] = \frac{(a + b[D]) + (c + d[D]) \exp \left( \frac{m[D] - C_m}{RT} \right)}{1 + \exp \left( \frac{m[D] - C_m}{RT} \right)} \tag{1}
\]

In Equation 1, [Signal] is either the ellipticity at 222 nm or fluorescence at 530 nm, a and c are the intercepts, b and d are the slopes of the base lines for native and unfolded species, and [D] and Cm are the concentration of urea for each experiment and the concentration at the midpoint of the reaction, respectively. For the transition curves at pH 8.0 and 25 °C, when reliable pre- and post-transition base lines could not be obtained because of a scarcity of data points, the base lines in accordance with other base lines were defined manually (see Fig. 1).

NMR Measurements—NMR spectra were measured at 40 °C on a Bruker DRX-800 spectrometer equipped with a cryogenic triple-resonance probe or a DRX-600 equipped with a triple axis-gradient triple resonance probe. Two-dimensional heteronuclear single quantum correlation (HSQC) spectra were
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RESULTS

Global Unfolding of FNR Induced by Urea—We characterized the global stability of FNR by measuring urea-induced unfolding under several conditions. First, the apparent unfolding transitions induced by 1 day of incubation at 25 and 40 °C at pH 8.0 in the presence of various concentrations of urea were measured with far UV CD and fluorescence of FAD (Fig. 1A). The far UV CD of FNR in its native state exhibited a spectrum typical of α/β proteins (data not shown; see Ref. 19) in accord with the x-ray crystallographic structure. The addition of high concentrations of urea resulted in global unfolding, providing a CD spectrum of a random coil state. The transition curves constructed from ellipticity at 222 nm were cooperative with the x-ray crystallographic structure. The transition curves con-structed from ellipticity at 222 nm were cooperative with the x-ray crystallographic structure. The transition curves constructed from ellipticity at 222 nm were cooperative with the x-ray crystallographic structure.

H/D exchange was performed in the presence and absence of 2 M urea at 40 °C and pD 8.0, and in the absence of urea at 40 °C and pD 6.0. Exchange was initiated by manually mixing lyophilized FNR with a 25 mM d-sodium phosphate buffer at pD 8.0. In the case of measurements at pD 6.0, a dilution method was applied where 30 μl of a 5 mM FNR solution dissolved in a 25 mM sodium phosphate buffer containing 50 mM sodium perchlorate at pH 6.4 was mixed with 270 μl of the same concentration of deuterium buffer at pD 6.0. Unless otherwise stated, all NMR measurements were carried out at 40 °C and, for the measurements at pD 6.0, 50 mM NaClO4 was added. The amide proton decays were monitored by measuring peak intensities in a series of two-dimensional 1H-15N HSQC spectra. The decay curves were fitted to a single exponential curve to obtain the apparent rate constant of exchange (k_app). The intrinsic rate constant (k_m) values were calculated with the SPHERE program (23).

H/D Exchange Monitored by NMR—The two-dimensional 15N-1H HSQC spectrum exhibited more than 314 amide group cross-peaks (Fig. 2A), of which 285 were assigned, accounting for ~95% assignments (excluding the N terminus and 13 Pro residues) (20). With these cross-peaks, we previously detected the protected amide protons by performing a H/D exchange for 3 days (20). Here, we carried out a far more detailed analysis of the H/D exchange kinetics, first, at pD 8.0 and 40 °C, where pD 8.0 and 40 °C, and 4 °C, where the catalytic activity of FNR is suppressed (15), exhibiting the same effects of incubation time on the apparent stability (Fig. 1D). The apparent transition curves showed that the stability is greater at pH 8.0 than at pH 6.0. In this research, to increase the solubility of FNR required for the NMR measurements, we added 50 mM sodium perchlorate (NaClO4) to the FNR solution at pH 6.0. Because NaClO4 is a chaotropic salt known to denature proteins at high salt concentrations (24), the unfolding in the presence of 50 mM NaClO4 (Fig. 1C) was also measured with the same incubation periods as in its absence. The results indicated that the apparent stability is slightly increased by a low concentration of NaClO4 as used in the present study. Although this might represent specific effects of the salt, the effects were too small to be addressed in this study.

Although the unfolding transitions of FNR were not reversible, we provisionally analyzed the transition curves on the basis of an equilibrium two-state mechanism. Consequently, the apparent parameters were obtained, including the free energy change of unfolding (ΔG_u) in the absence of denaturant (supple-mental Table S1).

FIGURE 1. Urea-induced unfolding transitions of FNR. A, unfolding curves were constructed from the ellipticity at 222 nm (○, ) and fluorescence of FAD at 530 nm (○, ) after incubation for 1 day at pH 8.0 and 25 (closed symbols) or 40 °C (open symbols). B–D, dependence of the unfolding transition curves on the incubation time observed with fluorescence at pH 8.0 (○) and pH 6.0 in the presence (○) and absence (●) of 50 mM NaClO4. The incubation times were 1 (○, ○), 3 (○, ○), and 7 (○, ○) days. All experiments were carried out at 40 °C. The solid lines indicate the transition curves fitted assuming a two-state mechanism and the broken lines represent the base lines derived from the fittings. Details are provided under “Experimental Procedures.”

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recorded with a spectral width of 12,820.5 Hz (1,024 complex points) for 1H and 2,439.0 Hz (128 complex points) for 15N dimensions with four or eight scans accumulated for each free induction decay for 1.3 h. The field frequency was locked at the deuterium signal from D2O. Spectra were processed using nmrPipe (22) and analyzed with nmrDraw, PIPP, and Sparky.

As expected for large proteins, the unfolding of FNR, made up of 314 amino acid residues and the cofactor FAD, was not reversible (19); the refolding yield after unfolding in a high concentration of urea (e.g. 8 M) was 10–30% depending on the methods and conditions used. Irreversible unfolding will lead the apparent transition curve to be dependent on the incubation time. With an increase in the period of incubation in urea, the concentration of urea required for unfolding decreased when the unfolding was monitored by the fluorescence of FAD (Fig. 1B). We also measured the unfolding transitions at pH 6.0 and 4 °C, where the catalytic activity of FNR is suppressed (15), exhibiting the same effects of incubation time on the apparent stability (Fig. 1D). The apparent transition curves showed that the stability is greater at pH 8.0 than at pH 6.0. In this research, to increase the solubility of FNR required for the NMR measurements, we added 50 mM sodium perchlorate (NaClO4) to the FNR solution at pH 6.0. Because NaClO4 is a chaotropic salt known to denature proteins at high salt concentrations (24), the unfolding in the presence of 50 mM NaClO4 (Fig. 1C) was also measured with the same incubation periods as in its absence. The results indicated that the apparent stability is slightly increased by a low concentration of NaClO4 as used in the present study. Although this might represent specific effects of the salt, the effects were too small to be addressed in this study.

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and making the first spectral measurement, many cross-peaks already disappeared (Fig. 2B). We confirmed that this rapid exchange was not caused by the dissolution of FNR (see below).

In contrast, even after 17 days of incubation, more than 40 peaks of strong intensity remained, indicating the presence of highly protected amide protons (Fig. 2C).

To accelerate the exchange, we added 2 M urea to the exchange buffer at pH 8.0 and 40 °C, by which the protein was destabilized but still retained the native conformation even after 7 days (Fig. 1B). The overall exchange was accelerated in 2 M urea, although we still observed many intense peaks even after 17 days (supplemental Fig. S1C). These highly protected residues included Cys42, Met60, Tyr78, Tyr95, Val115, Cys137, and Val145 in the FAD-binding domain and Leu168, Met184, Phe201, Leu258, Cys272, Asp298, and Asn310 in the NADP+ -binding domain (see Figs. 4 and 5 and supplemental Table S2).

These strongly protected residues were evidently located in the core secondary structure regions as shown by the x-ray structure, i.e. the six antiparallel β-strands and the α1-helix in the FAD-binding domain as well as the central β strands (β1, β2, and β4) of the β-sheet and α1-helix in the NADP+ -binding domain. Judging from the peak intensities for methyl protons, most of the FNR remained in the native state after 17 days in the presence of 2 M urea.

The activity of FNR in chloroplasts (i.e. photoreduction of NADP+) depends on the pH (15): whereas FNR is inactive at pH 6.0, it becomes more active with an increase of pH from 6.0 to

FIGURE 2. H/D exchange experiments of FNR at 40 °C. A, 1H-15N HSQC spectrum of uniformly 15N-labeled FNR measured in a 25 mM sodium phosphate buffer at pH 8.0. Assignments of the backbone amide signals are indicated by the respective single-letter codes and residue numbers. The assignments of the Trp side chain indoles are labeled w. B and C, 1H-15N HSQC spectra measured during the H/D exchange experiments in a 25 mM d-sodium phosphate buffer at pH 8.0. The spectra were acquired after 40 min (B) and 17 days (C) from the start of the exchange.

FIGURE 3. Exchange kinetics for the representative residues of FNR. Gly30 (A), Ala40 (B), Glu76 (C), Trp199 (D), Leu258 (E), and Glu312 (F) are shown. The peak intensities were plotted against the incubation time. The data were acquired at pH 8.0 in the presence (green) or absence (blue) of 2.0 M d-urea and at pH 6.0 (red). Open triangles at time 0 indicate the reference intensities measured in a H2O solution. The fitting curves are also shown assuming a single exponential function where the exchange rate constant (kex) and initial and final intensities were adjustable parameters.
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In all the H/D exchange experiments, the time ranges of exchanges widely varied depending on the residues. Generally, the reaction was faster in the order: pD 8.0 in 2 M urea > pD 8.0 in the absence of urea > pD 6.0. In the absence of urea at pD 8.0, among 269 assigned residues, 117 were exchanged completely within the first sampling period of 30 min, and are referred to as "the lower limit residues" (i.e. the protection is too low to quantify). In contrast, about 35 residues retained their initial peak intensities even after incubation for 17 days, and are referred to as "the upper limit residues" (i.e. the protection is too high to quantify). We excluded an additional 58 residues from the kinetic analysis, for which reliable data were not acquired because of a severe overlap of resonances or too large scattering of the peak intensity for unknown reasons, referring to them as "kinetics-undetermined residues." Consequently, the remaining 75 residues revealed a solid time-dependent decrease in peak intensity. For these residues, we assumed a first-order kinetics and fitted each time course to a single exponential curve, to estimate the apparent exchange rate constant (kex). Considering the presence of highly protected upper limit residues, the smallest kex one can determine here was about 2 × 10^{-6} min^{-1}, corresponding to 95% of intensity remaining after 17 days. On the other hand, the lower limit residues, with 5% of intensity left at 30 min, defined the largest kex to be about 0.1 min^{-1}.

Similar analyses were performed both at pD 8.0 in the presence of 2 M urea and at pD 6.0. The dead time measurements were 60 min at pD 8.0 in the presence of 2 M urea and 50 min at pD 6.0. The numbers of lower limit residues, residues that revealed the exchange rate constant, and upper limit residues were 128, 86, and 14, respectively, at pD 8.0 (in 2 M urea and 81, 98, and 49, respectively, at pD 6.0 (supplemental Figs. S1, S2, and Table S2). These numbers also indicate that the overall exchange was slower at pD 6.0, suggesting that the intrinsic exchange rate, which increases along with pH, is an important factor for determining the observed kinetics.

To take into account the variation in the exchange rate depending on the type of amino acid residue and neighboring residues, we calculated the protection factor (PF) for each res-
idue at pH 8.0 and 6.0. PF is defined as $k_{\text{int}}/k_{\text{ex}} = PF$, where $k_{\text{int}}$ is the exchange rate constant in the random coil conformation (23, 25). The profile of PF against the residue number shows significant variation ranging from $10^4$ to $10^{10}$ under the different conditions. Here, the lowest PF values to be determined assuming that 5% of peak intensity remains at the first time point are $10^4$–$10^5$ at pH 8.0 and $10^2$–$10^3$ at pH 6.0, where the range of PF is produced by the variation of $k_{\text{int}}$. For the same reason, the highest PF values to be determined with 95% of intensity remaining after an incubation period of 17 days at pH 8.0 are $10^8$–$10^{10}$ depending on the residues (supplemental Table S2). They are $10^6$–$10^8$ at pH 6.0 according to 95% remaining intensity after 23 days. In other words, the true PF values of lower limit residues and upper limit residues can be smaller and larger, respectively, than these limiting values.

**Protected Core Regions**—The plots of PF against the residue number (Fig. 4) and on the three-dimensional schematic structure of FNR (Fig. 5) showed that the protected residues are clustered in various regions, which are well correlated with the location of the core secondary structures. FNR consists of FAD- and NADP$^+$-binding domains. The protected residues at pH 8.0 were obviously clustered on the six antiparallel $\beta$ strands and one $\alpha$-helix in the FAD-binding domain and on the five parallel $\beta$ strands and two $\alpha$-helices in the NADP$^+$-binding domain. In contrast, loop regions between $\alpha$-helices and $\beta$ strands and N- and C-terminal regions were least protected from exchange. FNR has a characteristic N-terminal region composed of 30 amino acid residues that form unstructured conformation as indicated by x-ray (16) and NMR analyses (20). The results of H/D exchange showing no protected residue in this region were consistent with the x-ray structure, confirming the flexibility in the N-terminal region.

In the FAD-binding domain, $\beta_2$, $\beta_3$, and $\beta_4$ strands were the most strongly protected, forming the core of the $\beta$-barrel domain, whereas the $\alpha_1$-helix was also considerably well protected. In the NADP$^+$-binding domain, parallel $\beta_1$, $\beta_2$, and $\beta_4$ strands and $\alpha_1$-helix were strongly protected, indicating less extensively protected core regions in comparison with the cores of the FAD-binding domain. In particular, $\alpha_2$, $\alpha_3$, and $\alpha_5$ helices, forming the surface of the $\alpha/\beta$-fold of the NADP$^+$-binding domain, were only marginally protected. On the other hand, $\alpha_1$-helix of the NADP$^+$-binding domain, located at the interface between the two domains, was one of the most highly protected $\alpha$-helices in FNR. This bipartite protection pattern suggests that the NADP$^+$-binding domain consists of two subdomains with distinct conformational dynamics (NADP$^+$-rigid and NADP$^+$-flexible subdomains, see “Discussion”).

In the presence of 2 M urea at pH 8.0, PF decreased for many residues in comparison with values in the absence of urea (Figs. 4B and 5B). However, the extent of the acceleration of H/D exchange varied depending on the location of residues (Fig. 3): the residues in the NADP$^+$-binding domain tended to be exchanged faster than those in the FAD-binding domain, suggesting that the NADP$^+$-binding domain is preferentially destabilized in 2 M urea.

**pD-dependent Conformational Change—Intrinsic exchange** rates at pH 8.0 are 100-fold larger than those at pH 6.0. On the other hand, overall stability of FNR monitored based on CD or fluorescence measurements was slightly higher at pH 8.0 than at 6.0 (Fig. 1, supplemental Table S1). If the stability is independent of pH, the EX2 mechanism of H/D exchange (Ref. 10, see also “Discussion”) predicts that, whereas the apparent exchange rate increases by a factor of 100 when the pH value is increased from 6.0 to 8.0, the PF values remain constant. The observed protection patterns throughout the entire backbone of FNR were similar at pH 6.0 and 8.0 with the PF values at pH 8.0 slightly larger than those at pH 6.0, as expected from the greater stability at pH 8.0 (Fig. 4).

In our case, the EX2 mechanism would be the first assumption to be discussed because $k_{\text{int}}$ would be very large at both pH 8.0 values, even though the mechanisms of H/D exchange for FNR have not been established: not all the exchanged residues exhibited an ideal pH$^{-}$-dependent increase in the exchange rate (supplemental Table S2 see “Discussion”). On the other hand, careful inspection of the PF values indicated that the $\alpha_3$-helix, and the loops between $\alpha_5$–$\alpha_6$ and $\alpha_6$–$\beta_5$ in the NADP$^+$-binding domain were more protected at pH 6.0 than at pH 8.0: whereas many residues on the $\alpha_3$–$\alpha_6$ helices disappeared at the first point of the measurement (i.e., 40 min) at pH 8.0, their exchange kinetics could be followed at pH 6.0 revealing the PF values. This made the two subdomains of the NADP$^+$-binding domain distinguishable at pH 8.0, but unclear at pH 6.0.

**DISCUSSION**

**Protected Core Regions and Two Subdomains of NADP$^+$-binding Domain**—One of the most important results of the present study is that the H/D exchange at pH 8.0 revealed the presence of two subdomains in the NADP$^+$-binding domain: NADP$^+$-rigid and NADP$^+$-flexible subdomains. Bruns and Karplus (26) suggested the presence of three hydrophobic cores on the basis of the crystal structure of spinach leaf FNR, which are illustrated on the structure of maize leaf FNR (Fig. 5D). Cluster I located inside of the six-stranded $\beta$-barrel corresponds to the core of the FAD-binding domain. The NADP$^+$-binding domain contains two hydrophobic clusters, clusters II and III. Cluster II includes the interface between the two major domains and the N-terminal part of the NADP$^+$-binding domain. Cluster III, located on the far side of the parallel $\beta$-sheet, includes the C-terminal part of the NADP$^+$-binding domain. The profiles of PF were consistent with the hydrophobic clusters suggested by Bruns and Karplus (26) (Figs. 4 and 5) and, moreover, suggest the presence of two subdomains, each including one of these hydrophobic clusters. The NADP$^+$-flexible subdomain, rich in
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\(\alpha\)-helices, had more dynamic regions, as shown by Figs. 4 and 5, than the NADP\(^+\)-rigid subdomain, except for \(\beta_4\) strand.

Although the typical Rossman fold (18) is made of two structural mononucleotide binding motifs, i.e. a repeat of a \(\beta\)-\(\alpha\)-\(\beta\)-\(\alpha\)-\(\beta\) motif constituting a parallel six-stranded \(\beta\)-sheet with symmetrical \(\alpha\)-helices on both sides of the sheet with relative strand order \(\beta_1\beta_2\beta_3\beta_4\beta_5\beta_6\) (27), the NADP\(^+\)-binding domain of maize leaf FNR has a variant topology. To be more precise, the NADP\(^+\)-rigid subdomain retains pairs of the typical \(\beta\)-\(\alpha\)-\(\beta\)-\(\alpha\)-\(\beta\) motif (i.e. \(\beta_1\alpha_1\beta_2\alpha_2\beta_3\)) with relative strand order \(\beta_2\beta_3\beta_4\). On the other hand, the NADP\(^+\)-flexible subdomain has a \(\alpha_2\alpha_4\beta_5\alpha_6\alpha_7\) topology with relative strand order \(\beta_4\beta_5\), lacking one strand corresponding to \(\beta_6\), including two additional \(\alpha\)-helices (Fig. 4). There are several reports describing that the regions of the NADP\(^+\)-flexible subdomain is not the typical Rossman fold (26, 28), which are in accordance with our observation.

Our classification of the NADP\(^+\)-binding domain into two subdomains is strongly supported by limited proteolysis studies of spinach FNR, reporting that the NADP\(^+\)-flexible subdomain could be cleaved from the NADP\(^+\)-binding domain (29, 30). Two subdomains are connected by both hydrophobic interactions through the \(\beta_4\) strand and surface-exposed flexible loop region that contains a protease active site. In addition, the proposals that a domain should contain a hydrophobic core as an independent folding unit (31, 32) and that compactness without a hydrophobic core alone does not determine the presence of a domain (33) also provide us with reliable grounds for the division of the NADP\(^+\)-binding domain into the two subdomains.

**pH-dependent Change in Dynamics of the NADP\(^+\)-flexible Subdomain**—Because illumination of intact chloroplasts increases the stromal pH from about 6 to 8 (15), it has been suggested that the light-driven increase in pH induces a conformational change in FNR, which leads to the increase in its reductase activity and also in the affinity for its substrate, NADP\(^+\). Although a study using chemical modifications suggested the presence of the essential carboxyl group at the nucleotide-binding site (34), no detailed structure-based information in solution is available to date. The present study showed that the dynamics of the NADP\(^+\)-flexible subdomain, particularly of the four \(\alpha\)-helices, increased in response to a pD change from 6.0 to 8.0 (i.e. from night to daytime), suggesting that this elevated dynamics is responsible for the enhanced affinity of FNR for NADP\(^+\).

A large number of reports describing the consequences of increased dynamic motions in enzymes to their elevated activities are in accordance with our results. In the case of FNR, the most mobile part in a crystal structure revealed by the \(B\) factor falls into the NADP\(^+\)-flexible subdomain (26, 28). This part also corresponds to the structurally most varied regions among six distinct crystal structures of FNRs (28). Furthermore, two conformers of maize leaf FNR were found in a unit cell of the crystal prepared at pH 6.0. The \(B\) factors in the NADP\(^+\)-flexible subdomain in the two conformers differ considerably (Fig. 4D) (16), and this motional difference in crystals is consistent with the difference in our PF profiles in solution at pD 8.0 and 6.0. In addition, most of the residues of FNR that were largely perturbed in their chemical shifts upon binding of NADP\(^+\) at pH 8.0 existed in the NADP\(^+\)-flexible subdomain, suggesting that the motional mode is important for the substrate binding (Fig. 4E), whereas the largely perturbed residues upon formation of the Fd-FNR complex are mostly in the FAD-binding domain (Fig. 4E) (20).

The higher dynamic motion in the NADP\(^+\)-flexible subdomain at pD 8.0 than at pD 6.0 is most likely to facilitate rapid sampling in the conformational space for selecting the most suitable conformation for enhancing the binding affinity for NADP\(^+\), especially in the first binding step of the adenosine portion of NADP\(^+\) in the bipartite NADP\(^+\)-binding mode (20, 35). The fact that the presence of NADP\(^+\) at the active site is a prerequisite for the catalysis of FNR (35) can explain why the NADP\(^+\)-flexible subdomain exhibits the higher motional behavior than other regions of FNR at pD 8.0. Taken together, we postulate that binding of NADP\(^+\) to the NADP\(^+\)-flexible subdomain may trigger a change of motional mode in the Fd-binding site through a dynamic network, facilitating a higher affinity to Fd and increasing ultimately the overall catalytic cycle. Although it is not clear whether H/D exchange occurs through small scale motion (i.e. local fluctuation) or large scale motion (i.e. subglobal or global unfoldings), the different motional modes in the NADP\(^+\)-flexible subdomain at the two pD values probably control the binding affinity for NADP\(^+\). On the other hand, FNR needs to be stable during catalysis for sustaining its native structure. Therefore, rigid \(\beta_4\) strand seems important for maintaining stability of both the NADP\(^+\)-flexible subdomain as the small core and the NADP\(^+\)-rigid subdomain through hydrophobic interaction with \(\beta_4\) strand that is directly adjacent to the NADP\(^+\)-flexible subdomain in space (Fig. 5). Consequently, the structure of the NADP\(^+\)-flexible subdomain is dynamic in retaining the rigid small \(\beta_4\) strand. Our results are consistent with the fact that an enzyme needs to be both stable to maintain their native structures and flexible to allow conformational changes during catalysis (36). Considering evolution of enzymes for pH sensitivity, achieved by tuning the local motion (37), the pH-modulated change in motion of the NADP\(^+\)-flexible subdomain may be achieved through evolution for photosynthesis.

**Free Energy Change of Unfolding**—PFs obtained from the H/D exchange experiment can be related to the parameters of protein unfolding at each amide site, assuming a two-state transition model between the folded (N) and unfolded (U) states and the exchanged state (X),

\[
\begin{align*}
N & \rightarrow U \\
U & \rightarrow X
\end{align*}
\]

**SCHEME 1**

where \(k_U\) and \(k_R\) represent the unfolding and refolding rate constants, respectively (38, 39). The equilibrium constant \(K_{eq}\) between N and U is related to the microscopic rate constants by

\[
K_{eq} = \frac{k_U}{k_R}
\]

Under conditions where the conformational equilibrium is much faster than the intrinsic rate of exchange (i.e., \(k_R \gg k_{int}\) EX2 limit), the apparent exchange rate constant \(k_{ex}\) is represented by \(k_{ex} = K_{eq}k_{int}\). Here, PF corresponds to \(1/K_{eq}\) and \(\Delta G_{exx}\)
equals $RT \ln PF$. Under the other extreme condition where $k_{\text{int}}$ is much higher than the reflowing rate constant $k_{\text{ex}}$, $k_{\text{ex}}$ represents $k_{\text{lib}}$ (EX1 limit).

Generally for smaller proteins, assuming the EX2 mechanism (see “Results”), the highest $\Delta G_{\text{HX}}$ value estimated by H/D exchange corresponds to the overall $\Delta G_{\text{ex}}$ value calculated from the unfolding transition curve obtained with denaturants or with heat (39–42). On the other hand, there are some possible contributions to the increase in the $\Delta G_{\text{HX}}$ value, such as the existence of superprotected residues (43), the effects of solvent isotope (by $\pm 0–2$ kcal/mol), and proline isomerization (44, 45).

Although we do not know the true $\Delta G_{\text{lib}}$ for FNR, because the global unfolding is apparently irreversible, it is evident that conventional methods including analysis of the apparent unfolding transition curve significantly underestimate $\Delta G_{\text{ex}}$. Indeed, the $\Delta G_{\text{HX}}$ values of many residues were greater than the apparent $\Delta G_{\text{ex}}$ values obtained from CD or fluorescence measurements. Nevertheless, we could not determine the exact PF values for highly protected residues in the tight core regions and consequently the $\Delta G_{\text{HX}}$ value for global unfolding, suggesting only the lower limit of the real $\Delta G_{\text{HX}}$ value. Considering various unknown factors including the presence of superprotected residues and the solvent isotope effects enhancing the apparent stability of FNR, further studies are required to determine the exact correlation of global unfolding with the observed PF.

Conclusions—As far as we know, this is the largest protein for which a detailed analysis of H/D exchange has been performed, and the first site-specific study on the pH-modulated relationship between dynamics and function on FNR-type flavoenzyme in solution. We determined the motion of the molecule for each residue and its dependence on pH, revealing that the NADP$^+$ binding domain is made of two subdomains different in their dynamic behaviors. We suggest that the pH-dependent change in motion of the NADP$^+$-flexible subdomain is important for controlling the binding affinity of FNR for NADP$^+$, leading to the effective catalytic cycle of photosynthesis. The H/D exchange experiments provided the $\Delta G_{\text{HX}}$ values assuming the EX2 mechanism. Although the $\Delta G_{\text{HX}}$ values for highly protected residues are minimal and the real values might be larger, even defining the minimal values is of paramount importance for addressing the structural dynamics of large proteins for which no other approach is available at the moment.

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