Interaction of NADPH-Adrenoferredoxin Reductase with NADP+ and Adrenoferredoxin

EQUILIBRIUM AND DYNAMIC PROPERTIES INVESTIGATED BY PROTON NUCLEAR MAGNETIC RESONANCE*

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NADPH-adrenoferredoxin reductase, a flavoprotein from bovine adrenocortical mitochondria, has been investigated to elucidate the equilibrium and dynamic properties of the interaction with NADP+ and adrenoferredoxin (adrenodoxin) using proton NMR spectroscopy. The line width of the signals from NADP+ depends on the presence of the reductase. The off rate constant of NADP+ from the reductase is estimated to be about 15-20 s⁻¹ on the basis of line width measurements. No appreciable difference in off rate is detected between adenine and nicotinamide moieties of NADP+. Transferred nuclear Overhauser effect experiments for NADP+ indicate the time-dependent magnetization transfer profiles with a long lag phase. The proton NMR spectra during the titration of the reductase with adrenodoxin reveal that the reductase possesses distinct binding sites for both NADP+ and adrenodoxin. The sharp resonances in the aromatic region due to His-10 and His-62 of adrenodoxin were utilized as a probe to explore the interaction with the reductase. In the mixture of adrenodoxin and the reductase at the mol ratio of 6:1, T₁ values of the histidine residue in adrenodoxin were measured by the inversion recovery method. At low ionic strength, T₁ values of the resonances are not affected in the presence or absence of the reductase. In the presence of the reductase, T₁ values of resonances resulting from the histidine residues become shorter as the concentration of KCl increases because of rapid exchange between bound and free states. At low ionic strength (10 ms phosphate buffer), the off rate from the reductase is estimated to be less than about 4 s⁻¹. The off rate of adrenodoxin from the reductase could be the rate-limiting step in cytochrome c reductase activity at low ionic strength.

NADPH-adrenoferredoxin oxidoreductase (adrenodoxin reductase) is an FAD-containing flavoprotein with a molecular mass of 54,000. It is located on the inner membrane of adrenal mitochondria. The primary structure of bovine adrenodoxin reductase has been deduced from the nucleotide sequence of cDNA (2, 3). It functions in the mitochondrial electron transfer system supporting cytochrome P-450-dependent steroidogenic hydroxylation reactions (4). Adrenodoxin reductase delivers one reducing equivalents from NADPH at once and then delivers one reducing equivalent to adrenoferredoxin (adrenodoxin), an iron-sulfur protein containing a 2Fe-2S cluster. This two-to-one-electron step-down process via a semiquinone form was thought to be the major function of the electron transferring system composed of adrenodoxin reductase and adrenodoxin (5 and references therein). It was found that adrenodoxin forms a tight one-to-one complex with adrenodoxin reductase (6). At first, the complex of adrenodoxin and adrenodoxin reductase was regarded as an active species for electron transfer to cytochrome c. Later, Lambeth et al. (7) proposed that adrenodoxin transfers electrons as a mobile shuttle between adrenodoxin reductase and cytochrome P-450red (8).

The studies by chemical modification and oligonucleotide-directed mutagenesis of adrenodoxin proved that the binding sites on adrenodoxin for the reductase and cytochrome P-450red are nearly identical and are located in the sequence spanning the negatively charged amino acid residues between Asp-72 and Asp-86 (9-11). Recently, proton NMR studies of adrenodoxin demonstrated that a conformational change that occurred upon reduction of adrenodoxin was in the sequence of negatively charged amino acid residues assigned to the interaction site for the reductase partners (12, 13). The conformational change of adrenodoxin depending on its redox state could control the binding affinity of adrenodoxin for the reductase partners. This may provide a structural basis for adrenodoxin to work as a moving shuttle in the electron transfer mechanism.

Kinetic properties of adrenodoxin reductase in cytochrome c reductase activity have been explored extensively (8). It was shown that the maximum turnover number of the reductase (kcat) in adrenodoxin-mediated electron transfer to cytochrome c was about 10 s⁻¹ at the optimum conditions. The rate-limiting step in NADPH-dependent cytochrome c reductase activity was thought to be the electron transfer process from FAD of adrenodoxin reductase to the iron-sulfur cluster of adrenodoxin within the complex between adrenodoxin and the reductase (14). In contrast, an analogous electron transfer system, ferredoxin-NADP+ reductase and ferredoxin, exhibits a much larger turnover number in cytochrome c reductase activity (100 cytochrome c reduced per s) (15, 16). So far, however, no direct measurement showing the electron transferring rate within the complex has been reported.

The present study was conducted to elucidate the interaction of adrenodoxin reductase with adrenodoxin and NADP+ in dynamic and equilibrium states. The use of proton NMR spectroscopy for small molecules interacting with a macromolecule has provided some insight into their static and dynamic binding modes (17–21). We measured the proton NMR spectra for adrenodoxin and NADP+ in the presence of adrenodoxin reduc-
tase. The interactions of adrenodoxin and NADP⁺ with the reductase were characterized by measuring NOE(s),-T₁, values, and line widths of the resonances. The present results revealed the binding mode of NADP⁺ to the reductase and the dissociation rates of adrenodoxin and NADP⁺ from the reductase.

MATERIALS AND METHODS

Chemicals—NADP⁺ and NADPH were obtained from Sigma. Deuterated water and sodium 3-trimethylsilylpropionate-2,2,3,3-d₄ were purchased from MSD isotopes (Montreal, Canada). Other chemicals were of the highest quality obtained from Wako Pure Chemicals (Osaka, Japan).

Preparation of Adrenodoxin and Adrenodoxin Reductase—Bovine adrenodoxin and NADPH adrenodoxin reductase were prepared from bovine adrenocortical mitochondria. Adrenodoxin was prepared as described previously (22). Fractions with an absorption ratio at 414 nm and 280 nm (A₄14 nm/A₂80 nm) of 0.87-0.89 were collected and used for further experiments. Bovine NADPH-adrenodoxin reductase was prepared as reported previously (23). Adrenodoxin reductase with an absorption ratio at 260 nm and 450 nm (A₂60 nm/A₄50 nm) of 8.7-8.9 was obtained as the pure reductase. The purity of the protein was verified on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed according to the standard method of Laemmli (24). The concentrations of adrenodoxin and adrenodoxin reductase were determined using molar extinction coefficients of 9.8 mm⁻¹ cm⁻¹ at 414 nm and 11.3 mm⁻¹ cm⁻¹ at 450 nm, respectively (23).

Samples for ¹H NMR experiments were prepared by repeated concentration and dilution with 99.9% deuterated potassium phosphate buffer using the ultrafiltration membrane cone (Centrilo CF-25, Ami-co). The pH values of the samples were directly measured in the NMR sample tube sealed with a rubber septum by repeated evacuation and flushing with oxygen-free nitrogen gas. The titrations under nitrogen atmosphere were performed using an air-tight syringe washed with deoxygenated buffer under nitrogen gas.

¹H NMR Method—¹H NMR spectra were acquired with a JEOL GX-400 NMR spectrometer equipped with an array processor. The temperature of the sample was controlled by using a JEOL GVT temperature control unit. One-dimensional ¹H NMR spectra were recorded over 5,000 Hz with digital resolution of 0.61 Hz/point unless otherwise stated. An exponential line broadening of 1 Hz was applied to increase signal-to-noise ratio. The spin-lattice relaxation time (T₁) was measured using a inversion recovery pulse sequence. T₂ values were calculated by a nonlinear curve fitting method. Two-dimensional nuclear Overhauser effect spectra (NOESY) were obtained in the phase-sensitive mode with a mixing time of 150 ms (25, 26). Two-dimensional spectra were collected over 5,000 Hz with quadrature detection mode in both dimensions. For T₂ (evolution period) dimension the time proportional phase incrementation method (27) was employed. The carrier frequency was placed on the residual H₂PO₄⁻ signal. Solvent suppression for NOESY spectra was achieved through the observation channel with a DANTE (delays alternating with nutations for tailored excitation) pulse sequence (28). Normally, 32 scans were accumulated for each value of T₁, and 512 T₁ values were recorded with free induction decays of 2,048 complex points. Gaussian for T₂ (detection period) and shifted sine-bell for T₁ window functions were used for resolution enhancement. Spectra were zero filled to 2048 x 1024 points with digital resolution of 2.44 Hz/point on T₂ and 4.9 Hz/point on T₁. All spectra were referenced to internal sodium 3-trimethylsilylpropionate-2,2,3,3-d₄.

The time-dependent transferred NOE on the proton resonances of the substrate was measured using selective irradiation of the resonances from the free substrate (29, 30). Irradiation power was attenuated down to 32 decibels below 0.5 watt but was enough to saturate the interested resonance within 30 ms. The ratio of the resonance intensity with control irradiation at 2.25 ppm, Ic(t), was also measured as a function of irradiation time. The ratio between them was expressed as the relative intensity.

RESULTS

Adrenodoxin reductase is a membrane-associated flavoprotein with a relatively large molecular mass. There has been reported little ¹H NMR work for the reductase. Thus, we first measured the ¹H NMR spectra of the reductase and characterized their pH⁺-dependent spectral changes. The ¹H NMR spectra of adrenodoxin reductase for the aromatic region are shown in Fig. 1A. The chemical shifts of the annotated signals in Fig. 1A during the pH⁺ titration are plotted in Fig. 1B. Relatively sharp resonances except signals B and F shifted downfield as the pH decreased. These resonances showing pH-dependent chemical shift could be tentatively assigned to the C2H or C4H protons of histidine residues.

Interaction of NADP⁺ with Adrenodoxin Reductase—¹H NMR spectra of the reductase during the titration of the reductase with NADP⁺ indicate no major spectral changes in the spectral region for the aromatic resonances. No signals arising from NADP⁺ appear in the spectra until more than the stoichi-
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During the titration of the reductase with more than the stoichiometric amount of NADP+, the line width of the resonances due to NADP+ get sharper as the concentration of NADP+ increases. Under the fast exchange condition, the observed line widths of the substrate are the weighted average of the line widths for bound and free substrates. As the resonances due to bound NADP+ are excessively broadened beyond detection, averaging of the line width between free and bound NADP+ in the sample of the reductase with 1.3 eq of NADP+ causes extensive broadening of the resonances from free NADP+. The observed line width of the resonances from free NADP+ indicates no appreciable averaging with those of bound NADP+, suggesting that the exchange rate is enough slower than the line width of bound NADP+. Thus, under the slow exchange condition, the line width depending on the mol ratio of NADP+ to the reductase could be due to the life time broadening. As the Kᵣ of NADP+ is much lower than the concentration employed in the present experimental conditions, the binding site for NADP+ is fully occupied. Thus, the life time of free NADP+ is dominated by the off rate constant (kₐff) of NADP+ from the reductase. Under these conditions, the observed line widths at the various concentrations of NADP+ are described as follows (32).

\[
\frac{1}{T_{20}} = \frac{1}{T_{2\text{free}}} + \frac{k_{\text{eff}}}{x - 1}
\]

(Eq. 1)

where \(T_{20}\) is the observed spin-spin relaxation time, \(T_{2\text{free}}\) is the spin-spin relaxation time of free NADP+, and \(x\) is the mol ratio of NADP+ to the reductase. The estimated off rates of NADP+ from the reductase are calculated from the line width of N2H proton for nicotinamide moiety and A8H proton for adenine moiety. The off rates are plotted as a function of the mol ratio between NADP+ and the reductase in Fig. 2B. The calculated kₐff remains constant over a wide range of the mol ratio examined, suggesting that the equation is valid over the condition employed and that no other significant effects interfere. The resonances from both adenine and nicotinamide moieties of NADP+ indicate a similar off rate of about 15–20 s⁻¹. No significant difference in the off rates between nicotinamide and adenine moieties of NADP+ was found.

NOE Measurement—NOESY spectra for NADP+ in the phase-sensitive mode were measured in the presence of adrenodoxin reductase. Fig. 3 shows a two-dimensional phase-sensitive NOESY spectrum with a waiting time of 150 ms. Negative NOE ratios were observed between the N1'H/N2H and N2'H/N6H protons for nicotinamide-ribose moiety of NADP+, and NOEs between the A2'H/A8H protons and a faint signal between the A1'H/A8H protons were observed for adenosine-ribose moiety. These negative NOEs were not observed in the system without adrenodoxin reductase (data not shown), indicating that the observed NOESY cross-peaks in Fig. 3 arose due to the magnetization transfer within the NADP+ molecule that was complexed with the reductase. To the contrary, NADP+ did not ex-
hibit any negative NOESY cross-peaks, even in the presence of adrenodoxin reductase (data not shown). This could be due to the low affinity of the reductase for NADH ($K_m = 5.56$ mm) as determined in cytochrome $c$ reductase activity (6).

Time-dependent transferred NOE between the specific pairs of the resonances were measured by one-dimensional NOE. Only resonances with cross-peaks in NOESY spectrum show time-dependent negative NOEs (data not shown). Time-dependent profiles for development of NOE exhibit a lag phase of as long as $80$ ms. Usually, a long lag phase was observed in such a system with a slow cross-relaxation rate. This was thought of as an indication of the spin diffusion, which is no longer directly connected with the distance between the specific pair of protons (29, 30, 33). However, the observed negative NOEs are highly specific for the pairs of resonances that form cross-peaks in the NOESY spectrum. Recent simulation demonstrated the exchange lag phase for the system with an intermediate exchange rate comparable with the cross-relaxation rate (19). This implies that consistent with the off rate of NADP$^+$ from the reductase determined in this work.

Unfortunately, we failed to determine the interproton distances from the data for time-dependent transferred NOE measurement. Still, it is possible to estimate qualitatively the conformation of the bound NADP$^+$ from the NOESY spectrum. Within the adenine-ribose moiety of bound NADP$^+$, the intense NOESY cross-peak between $A_2'H/A8H$ protons and the weaker signal between $A1'H/A8H$ protons suggests the adenine-ribose glycosidic torsional angle to be an anticonformation. Likewise, the NOESY cross-peaks between $N1'H/N2'H$ and $N2'H/N6H$ protons for the nicotinamide-ribose moiety confine the nicotinamide-ribose glycosidic torsional angle to an anticonformation.

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$^1$H NMR spectra during the titration of the reductase with adrenodoxin are shown in Fig. 4. Several of the resonances of the reductase (resonances $A$, $I$, and $J$ in Fig. 1A) are broadened and/or shifted out upon the complex formation with adrenodoxin. As the stoichiometric amount of adrenodoxin was added, these resonances were completely abolished on the spectrum. This indicates that the interaction between adrenodoxin and the reductase is highly specific and that a tight one-to-one complex is formed between them. It is interesting to note that the resonance $J$ at $8.75$ ppm is broadened away not only upon complex formation with adrenodoxin but also upon reduction of FAD with sodium dithionite.

Noncompetitive binding of adrenodoxin and NADP$^+$ for adrenodoxin reductase is demonstrated in Fig. 5. In the system with the reductase and $2$ eq of NADP$^+$, further addition of adrenodoxin abolished the above mentioned signals arising from the reductase without changing the signal intensity of the resonances due to NADP$^+$. This indicates that the reductase does not release NADP$^+$ upon complex formation with adrenodoxin, suggesting that the reductase possesses distinct binding sites for both NADP$^+$ and adrenodoxin. Close inspection of the spectra further reveals that the line width of the resonances due to free NADP$^+$ becomes sharper upon complex formation with adrenodoxin. This may imply that binding of adrenodoxin to the reductase slows the off rate of NADP$^+$ from the reductase.

Nonselective $T_1$ values for the $C2H$ protons of His-10 and His-62 residues in adrenodoxin were measured in the system containing adrenodoxin reductase and adrenodoxin. The complex formation with the reductase affects the relaxation time of these resonances. Chemical modification of adrenodoxin with diethyl pyrocarbonate indicated that the side residues of His-10 and His-62 were not directly involved in the site for interaction with redox partners (13). Thus, the effects on the relaxation time could be due to the change in the rotational correlation time upon complex formation. In the presence of the reductase at the concentration of one-sixth of adrenodoxin, $T_1$ values of histidine residues in adrenodoxin become shorter as the concentration of NaCl increases. On the other hand, without adrenodoxin reductase, the addition of $0.2$ mm NaCl does not change $T_1$ values for the histidine residues significantly (Fig. 6).

At low NaCl concentration, $T_1$ values of histidine residues in
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Adrenodoxin reductase transfers electrons from NADPH to adrenodoxin. Dynamic and equilibrium properties of the interaction among adrenodoxin reductase and its redox partners were monitored through measuring the 1H NMR spectra for NADP⁺ and adrenodoxin. Titration of the reductase with adrenodoxin and NADP⁺ on the 1H NMR spectra indicates that they form a stable ternary complex among them. The k_off of NADP⁺ from the complex with the reductase is shown to be as slow as 15–20 s⁻¹. This could be the reason for a long lag phase found in time-dependent transferred NOE measurement (19). The slow off rate of NADP⁺ from the complex with adrenodoxin reductase prevented us from reliably calculating the distance between the specific protons. Fig. 7 is a schematic presentation of the ternary complex among the reductase, NADP⁺, and adrenodoxin. Asp-76 and Asp-79 of adrenodoxin have been shown to be located in the interface for protein-protein interaction and to participate in the electrostatic interaction with the reductase (10). The resonance J at 8.75 ppm (Fig. 1) is probably located at the interaction site with adrenodoxin and is also close to the isoalloxazine ring of FAD.

**DISCUSSION**

Adrenodoxin reductase transfers electrons from NADPH to adrenodoxin. Dynamic and equilibrium properties of the interaction among adrenodoxin reductase and its redox partners were monitored through measuring the 1H NMR spectra for NADP⁺ and adrenodoxin. Titration of the reductase with adrenodoxin and NADP⁺ on the 1H NMR spectra indicates that they form a stable ternary complex among them. The k_off of NADP⁺ from the complex with the reductase is shown to be as slow as 15–20 s⁻¹. This could be the reason for a long lag phase found in time-dependent transferred NOE measurement (19). The slow off rate of NADP⁺ from the complex with adrenodoxin reductase prevented us from reliably calculating the distance between the specific protons. Fig. 7 is a schematic presentation of the ternary complex among the reductase, NADP⁺, and adrenodoxin. Asp-76 and Asp-79 of adrenodoxin have been shown to be located in the interface for protein-protein interaction and to participate in the electrostatic interaction with the reductase (10). The resonance J at 8.75 ppm (Fig. 1) is probably located at the interaction site with adrenodoxin and is also close to the isoalloxazine ring of FAD.

**Qualitative interpretation of the NOESY spectrum demonstrates that the nicotinamide-ribose glycosidic torsional angle is an anticonformation in the binary complex with adrenodoxin reductase. Light and Walsh (34) investigated the stereochemistry of hydrogen transfer using [(S)-2H,NAD⁺]⁻ and observed a large isotope effect, indicating that the bond between the N4 carbon and the pro-S hydrogen is broken upon reduction of adrenodoxin reductase with NADPH. This implies that the pro-S hydrogen of a nicotinamide ring faces on top of an isoalloxazine ring of FAD with an anti-nicotinamide-ribose glycosidic torsional angle. Karplus et al. (35) determined the three-dimensional structure of spinach ferredoxin-NADP⁺ reductase by x-ray crystallography. They revealed the binding mode of 2'-phosphoadenosine monophosphate and further ex-

**Fig. 5.** 1H NMR spectra of adrenodoxin reductase with 2 eq of NADP⁺ in the presence (upper trace) and absence (lower trace) of a stoichiometric amount of adrenodoxin. Conditions are the same as in Fig. 4. Shaded areas in the lower trace were abolished upon addition of adrenodoxin.

**Fig. 6.** Spin-lattice relaxation times of the resonances for histidine residues of adrenodoxin in the presence and absence of adrenodoxin reductase are plotted as a function of the concentration of added NaCl. ○ denotes the relaxation times for C2H proton of His-62 in the presence of the reductase, and Δ is for those without the reductase. ● is for His-10 with the reductase and Δ without the reductase. The samples with adrenodoxin reductase contained 1.2 mM adrenodoxin and 0.2 mM adrenodoxin reductase in 10 mM phosphate buffer, pH 7.5, at 28 °C.

adrenodoxin are not affected by the presence of adrenodoxin reductase. Increasing ionic strength shortened the T1 values only in the presence of the reductase. This implies that the rate for the protein-protein interaction between adrenodoxin and the reductase is modulated by the ionic strength of the medium. Under fast exchange conditions, the observed spin-lattice relaxation rate (1/T1) is a weighted average of those for bound and free states, as expressed in the following equation

\[
\frac{1}{T_1} = \frac{1}{T_{1b}} + \frac{5}{T_{1f}}
\]

where \(T_{1b}\) is the observed spin-lattice relaxation time, \(T_{1b}\) and \(T_{1f}\) are those for bound and free states, respectively. In contrast, under slow exchange conditions, the observed spin-lattice relaxation time is expected to exhibit biphasic time dependence, the fast phase from adrenodoxin in bound state and the slow phase from free adrenodoxin. Adrenodoxin in complex with the reductase contributes to only one-sixth of the observed signal intensity. Furthermore, as the line width of the signal from adrenodoxin in the bound state is much broader than that of free adrenodoxin, its contribution to the signal intensity measured as the signal height must be even smaller. Actually, the semilogarithmic plots of the signal intensities were apparently monophasic under these experimental conditions. Assuming the slow exchange condition at low ionic strength, the upper limit of the off rate is estimated to be less than \(6/T_{1F}\), which is approximately equal to 4 s⁻¹. The estimated off rate constant is comparable with the \(k_{on}\) in cytochrome c reductase activity at low ionic strength. In the presence of 0.2 M NaCl, assumption of the fast exchange condition allows us to estimate the \(T_{1b}\) to be about 0.6 s, which is comparable with \(T_1\) of the spectral envelope of the reductase complexed with a stoichiometric amount of adrenodoxin at low ionic strength. This favored our explanation for the present results of relaxation time.

**Fig. 7.** Schematic representation of the ternary complex among adrenodoxin reductase, NADP⁺, and adrenodoxin at low ionic strength. Adrenodoxin reductase possesses distinct binding sites for both NADP⁺ and adrenodoxin. The \(k_{off}\) of adrenodoxin is estimated to be less than 4 s⁻¹. The \(k_{off}\) of NADP⁺ is determined to be about 15–20 s⁻¹. The isoalloxazine ring of FAD is located at the site close to both NADP⁺ and adrenodoxin.
tended the discussion on a hypothetical complex with NADPH, which indicates that pro-R hydrogen of a nicotinamide faces on top of an isoalloxazine ring with an anti-nicotinamide-ribose glycosidic torsional angle. This conformation is in agreement with the stereospecificity of the reaction mediated by ferredoxin-NADP⁺ reductase. The ferredoxin-NADP⁺ reductase structure with 2'-phosphoadenosine monophosphate was compared with the flavine and NADPH from glutathione reductase after the two flavines had been superimposed, showing that the nicotinamide from glutathione reductase approaches FAD from the opposite direction of that in ferredoxin-NADP⁺ reductase. Glutathione reductase catalyzes the pro-S hydrogen-specific transfer with an anti-nicotinamide-ribose moiety of NADP⁺ in adrenodoxin reductase is similar to that in glutathione reductase rather than that in ferredoxin-NADP⁺ reductase, which is long believed to be analogous to adrenodoxin reductase. This was regarded to be a requisite condition in order that the “shuttle mechanism” works properly. Actually, they showed that the first order rate constant for dissociation of the complex between adrenodoxin and the reductase was approximately 300 s⁻¹ and was independent of ionic strength. We have no definite explanation for this discrepancy at this moment. Even though the off rate of adrenodoxin from the complex with the reductase is the rate-limiting step in this electron transfer system, the electron transfer mechanism, in which adrenodoxin transfers electrons as a moving shuttle, is still valid and the most plausible.

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