Interaction of NADPH−Adrenodoxin Reductase with $NADP^+$ as Studied by Pulse Radiolysis[†]

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ABSTRACT: The reduction of flavin in NADH−adrenodoxin reductase by the hydrated electron (e_{aq}^-) was investigated by pulse radiolysis. The e_{aq}^- reduced directly the flavin of the reductase to form a blue semiquinone of the enzyme. Subsequently, the semiquinone decayed by dismutation to form the oxidized and fully reduced forms of the enzyme with a second-order rate constant of 4.4×10^4 M^{-1} s^{-1}. In the presence of equimolar $NADP^+$, the decay of e_{aq}^- accompanied an absorption increase at 400 nm, the spectrum of which, formed transiently, is identical to that of NADP radical ($NADP^•$). Subsequently, the transient species decayed concomitantly with the formation of the semiquinone. The rate constant in the formation of the semiquinone was independent of the concentration of the enzyme (6.1×10^4 s^{-1} at pH 7.5). From these results, it is concluded that e_{aq}^- reacts with $NADP^+$ bound to the enzyme to form $NADP^•$ initially, and subsequently, an electron flows from the $NADP^•$ to the flavin by an intracomplex electron transfer. A similar result was obtained in the reaction of CO_2^- or *N*-methylnicotinamide radical with the $NADP^+$−adrenodoxin reductase complex. These results suggest that the nicotinamide moiety of $NADP^+$ bound to the enzyme is accessible to the solvent and masks the flavin completely.

Adrenodoxin oxidoreductase (NADPH:adrenal ferredoxin adrenodoxin reductase, EC 1.18.1.2) is an FAD-containing flavoprotein which is an essential component of the electron transport system for the cytochrome P-450-dependent hydroxylation (Omura et al., 1966). Adrenodoxin reductase receives two electrons from NADPH and then delivers one electron to adrenodoxin, an iron−sulfur protein containing a 2Fe−2S cluster. The molecular mechanism of electron transfer from NADPH to cytochrome P-450 in the adrenal hydroxylating system via adrenodoxin reductase has been studied. For the electron transport to cytochrome P-450, shuttling of adrenodoxin between adrenodoxin reductase and cytochrome P-450 (Lambeth et al., 1979) or ternary complex formation among them was proposed (Kido & Kimura, 1979). A semiquinone form of adrenodoxin reductase has not been identified clearly through the catalytic cycle, though the two- to one-electron step-down process via the semiquinone form was thought to be a major function of adrenodoxin reductase (Kamin & Lambeth, 1982).

Adrenodoxin reductase was reduced completely by two reducing equivalents without formation of the stable semiquinone when it was titrated anaerobically with sodium dithionite (Chu & Kimura, 1973; Lambeth & Kamin, 1976, 1977). The titration of the reductase by NADPH yields a charge-transfer complex, which was designated as a complex of the reduced enzyme and $NADP^+$ (Chu & Kimura, 1973). On the other hand, the semiquinone was formed by anaerobic reduction of adrenodoxin reductase with more than the stoichiometric amount of NADPH (Kitagawa et al., 1982; Sakamoto et al., 1982). Nonaka et al. (1986) have proposed that the semiquinone is stabilized by binding of NADPH and that the complex of the semiquinone and NADPH and/or the complex of the fully reduced form and NADPH are the true active species in the catalytic cycles for P-450's reduction via adrenodoxin in the presence of excess NADPH.

Some of the advantages of the pulse radiolysis technique for determination of spectral and kinetic behavior of one-electron reduction products of flavoproteins have been demonstrated (Faraggi & Klapper, 1979; Klapper & Faraggi, 1983; Kobayashi et al., 1983, 1988, 1993; Anderson et al., 1986). By using this technique, we succeeded in directly observing a semiquinone of cytochrome b_5 reductase, which cannot be observed as a stable form. The present paper describes the reduction of the flavin of adrenodoxin reductase by hydrated electron (e_{aq}^-)[1] in the presence and absence of $NADP^+$, results of which are compared with those of cytochrome b_5 reductase (Kobayashi et al., 1988). Unlike cytochrome b_5 reductase, e_{aq}^- or CO_2^- reacted bound $NADP^+$ in the equimolar complex with adrenodoxin reductase, and subsequent electron flow to the flavin by intracomplex dominates the rate of the reduction of the flavin. Part of this work has been presented in a preliminary form (Miki et al., 1994).

MATERIALS AND METHODS

Chemicals. $NADP^+$, NAD^+, and *N*-methylnicotinamide were obtained from Sigma. All other reagents were of the

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[1] Abbreviations: e_{aq}^-, hydrated electron; NMA, *N*-methylnicotinamide; E-FAD, oxidized adrenodoxin reductase; $E\text{-}FADH^•$, neutral radical adrenodoxin reductase; $E\text{-}FADH^-$, fully reduced adrenodoxin reductase.

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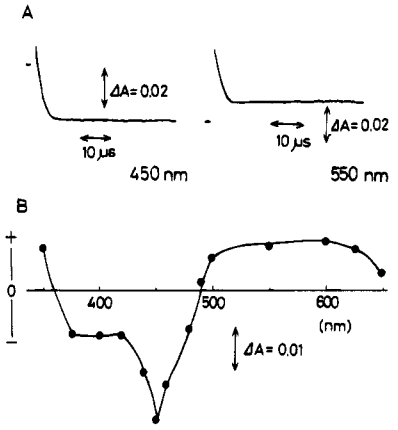


FIGURE 1: (A) Absorption changes after pulse radiolysis of adrenodoxin reductase measured at 450 and 550 nm. The reaction medium contained 41 μM enzyme, 0.1 M *tert*-butyl alcohol, and 10 mM phosphate buffer (pH 7.4). (B) Kinetic difference spectrum at 20 μs after the pulse.

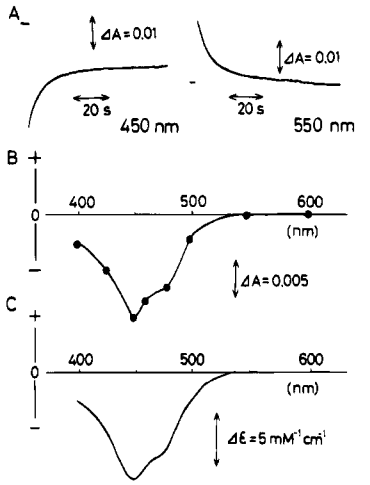


FIGURE 2: (A) Absorption changes after pulse radiolysis of adrenodoxin reductase measured at 450 and 550 nm. The experimental condition is the same as in Figure 1. (B) Kinetic difference spectrum at 30 s after the pulse. (C) Difference spectrum of the fully reduced form minus the oxidized form of the enzyme. The fully reduced form of the enzyme was obtained by the addition of dithionite.

highest grade of purity available and were obtained from a commercial source.

Preparation of Adrenodoxin and Adrenodoxin Reductase. Bovine adrenodoxin and NADPH−adrenodoxin reductase were prepared from bovine adrenocortical mitochondria. Adrenodoxin was prepared as described previously (Hiwatashi et al., 1986). Fractions with an absorption ratio at 414 and 280 nm ($A_{414\ nm}/A_{280\ nm}$) of 0.87−0.89 were collected and used for further experiments. Bovine adrenodoxin reductase was prepared as reported previously (Hiwatashi et al., 1976). The enzyme with an absorption ratio at 260 and 450 nm ($A_{260\ nm}/A_{450\ nm}$) of 8.7−8.9 was obtained as the pure reductase. 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 (Hiwatashi et al., 1986).

Pulse Radiolysis. A sample of adrenodoxin reductase for pulse radiolysis was prepared as follows. Argon gas was passed over the surface of the solution of the enzyme containing 10 mM phosphate buffer, pH 7.5, and 0.1 M *tert*-butyl alcohol for scavenging the $OH^{\bullet}$ with stirring. No effect of *tert*-butyl alcohol (0.1 M) on the optical absorption spectrum of the enzyme was seen. To study the reaction of CO_2^-, the samples containing 0.1 M sodium formate were deaerated by repeated evacuation and flushing with argon, and then 1 atom of N_2O gas was introduced anaerobically. A fresh solution was used for each pulse. Pulse radiolysis experiments were performed with an electron linear accelerator for the Institute of Scientific and Industrial Research, Osaka University (Kobayashi et al., 1988, 1993). The pulse width and energy were 8 ns and 27 MeV, respectively. The light source was a 150 W halogen lamp or 1-kW xenon lamp. When the light was passed through an optical path, the transmitted light intensities were analyzed and monitored by a fast spectrophotometric system composed a Nikon monochromator, an R-928 photomultiplier, and a Unisoku data-analyzing system.

RESULTS

The reduction of flavin in adrenodoxin reductase by e_{aq}^- was investigated by pulse radiolysis. The decay of e_{aq}^- accompanied an absorption decrease at 450 nm and an absorption increase at 550 nm characteristic of a blue semiquinone of the enzyme, as shown in Figure 1A. Figure 1B shows the kinetic difference spectrum 20 μs obtained after pulse radiolysis. The spectrum shows that the blue semiquinone is formed in the reaction of e_{aq}^- with FAD of adrenodoxin reductase, as shown in reaction 1.

$$e_{aq}^- + \text{E-FAD} \xrightarrow{H^+} \text{E-FADH}^{\bullet} \qquad (1)$$

Subsequently, the semiquinone was found to decay in the time range of seconds. Typical examples are shown in Figure 2A. The absorption at 450 nm increased, and that at 550 nm decreased. Figure 2B shows the kinetic difference spectrum at 30 s after the pulse, and the spectrum is essentially identical to that of the oxidized form minus the reduced form of the enzyme in Figure 2C. The half-life of the semiquinone was affected by the concentration of the semiquinone generated initially after the pulse, not that of the oxidized enzyme in the sample. This suggests that the semiquinone decays with a bimolecular reaction. In fact, the decrease of absorbance at 550 nm obeyed second-order kinetics. Therefore, it is concluded that the process in Figure 2A can be attributed to the dismutation of the semiquinone which forms the oxidized and fully reduced forms of the enzyme in reaction 2.

$$2\text{E-FADH}^{\bullet} \rightarrow \text{E-FAD} + \text{E-FADH}^- + \text{H}^+ \qquad (2)$$

The second-order rate constant of reaction 2 is estimated to be 4.4×10^4 M^{-1} s^{-1}. A similar process was observed in the case of cytochrome b_5 reductase (Kobayashi et al., 1988).

The reduction of adrenodoxin reductase with e_{aq}^- was performed in the presence of equimolar $NADP^+$. Under the condition, nearly all of $NADP^+$ is bound to the enzyme, using the dissociation constant of 5.32 μM (Chu & Kimura, 1973; Nonaka et al., 1986). Typical examples are shown in Figure 3A. Though the absorption at 800 nm due to the e_{aq}^- decayed with a half-time of 1 μs, the reduction of the flavin occurred in the time range of 10 μs. The time-resolved kinetic difference spectra after the pulse are shown in Figure 3B. A transient species with an absorption maximum at 400 nm was formed at 2 μs after the pulse. The spectrum is identical to that obtained upon pulse radiolysis of free NAD^+

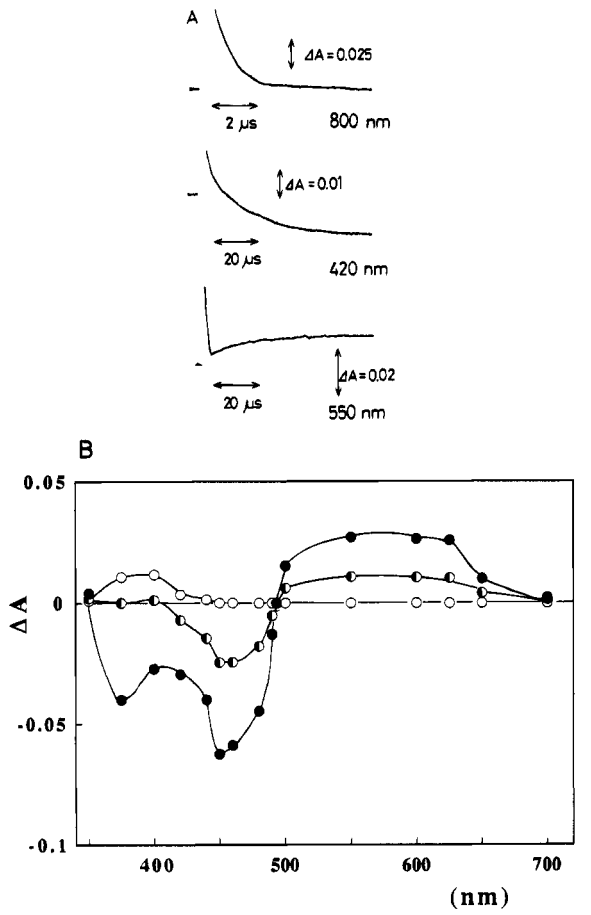
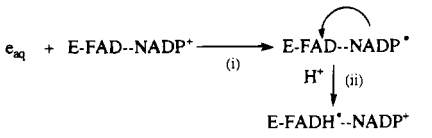


FIGURE 3: (A) Absorption changes after pulse radiolysis of $NADP^+$—adrenodoxin reductase measured at 800, 420, and 550 nm. The reaction medium contained 80 μM enzyme, 80 μM $NADP^+$, 0.1 M *tert*-butyl alcohol, and 10 mM phosphate buffer (pH 7.4). (B) Kinetic difference spectra of pulse radiolysis of adrenodoxin reductase. The spectra were taken at 2 μs (○), 10 μs (◐), and 50 μs (●) after pulse radiolysis. The reaction medium contained 40 μM enzyme, 40 μM $NADP^+$, 0.1 M *tert*-butyl alcohol, and 10 mM phosphate buffer (pH 7.4).

in aqueous solution (Land & Swallow, 1968). Then, the decay of the transient species accompanied an absorption decrease at 450 nm and an absorption increase at 550 nm. As judged from the resulting spectrum obtained at 50 μs after the pulse, the blue semiquinone is formed in the reaction. In this system, 5.6 μM $NADP^•$ (determined by calculation using an extinction coefficient of 2.1 mM^{-1} cm^{-1} at 400 nm) (Land & Swallow, 1968) was generated initially, and subsequently, the 5.4 μM enzyme was reduced. This indicates that almost stoichiometric reduction of the flavin by $NADP^•$ occurs in this system.

The reaction of e_{aq}^- with $NADP^+$—adrenodoxin reductase obeyed pseudo-first-order kinetics, when the 0.5−8 μM e_{aq}^- was generated in a solution containing 20−100 μM adrenodoxin reductase. The apparent first-order rate constant determined by the decay of e_{aq}^- at 800 nm increased with an increase of the concentration of the enzyme. The second-order rate constant for the reaction of e_{aq}^- is calculated to be 1×10^{10} M^{-1} s^{-1}. On the other hand, the rate constant for the formation of the semiquinone was independent of the concentration of the enzyme within experimental error. From these results, the reaction sequence following pulse radiolysis of the adrenodoxin reductase—$NADP^+$ complex can be described by Scheme 1. The e_{aq}^- reacts with $NADP^+$ bound to the enzyme to form $NADP^•$ (step i), and the $NADP^•$

Scheme 1



transfers an electron to FAD by intramolecular migration with a first-order rate constant of 6.1×10^4 s^{-1} (step ii). In this scheme, the reduction of FAD is not due to a bimolecular reaction between free $NADP^•$ in the bulk solution and FAD of the enzyme, since the rate constant of the reduction of FAD was independent of the concentration of the enzyme.

The semiquinone thus formed decayed to form the mixture of the oxidized and fully reduced enzymes, as observed in the absence of $NADP^+$ (data not shown). The rate of the dismutation was not dependent on the concentration of $NADP^+$, suggesting that $NADP^+$ binding to the enzyme does not influence this process.

In the presence of 0.1 M HCO_2^-, CO_2^- is produced via reactions 3 and 4 by pulse radiolysis of N_2O-saturated aqueous solution.

$$e_{aq}^- + N_2O + H_2O \rightarrow OH^• + OH^- + N_2 \quad (3)$$

$$OH^• + HCO_2^- \rightarrow CO_2^- + H_2O \quad (4)$$

Figure 4A shows the absorption changes after pulse radiolysis of adrenodoxin reductase in the presence of HCO_2^- and N_2O. The absorption decreased at 450 nm and increased at 550 nm, indicating that CO_2^- reduces the flavin of the enzyme effectively. The second-order rate constant for the reaction of CO_2^- with the enzyme is estimated to be 2.9×10^8 M^{-1} s^{-1} at pH 7.5, when 0.8 μM CO_2^- was generated in solutions containing 24−86 μM enzyme. Similarly, the absorption changes due to the reaction of CO_2^- with $NADP^+$—adrenodoxin reductase complex were observed (Figure 4B). Unlike $NADP^+$-free enzyme, the rate constant of the formation of the semiquinone was independent of the concentration of the enzyme (data not shown). Moreover, $NADP^•$ was generated prior to the formation of the semiquinone (Figure 4C), as observed in the reaction of e_{aq}^-. The rate of the formation of $NADP^•$ was dependent on the concentration of the enzyme. This indicates that CO_2^- reacts with $NADP^+$ bound to the enzyme initially, where the second-order rate constant in this process is estimated to be 1.2×10^9 M^{-1} s^{-1}. Subsequently, intramolecular electron transfer from the $NADP^•$ to the flavin of the enzyme occurs with a first-order rate constant of 6.1×10^4 s^{-1}.

The reaction of adrenodoxin reductase with e_{aq}^- was performed in the presence of 1 mM $NADP^+$, NAD^+, or *N*-methylnicotinamide (NMA). Figure 5 compares the absorption changes at 450 nm after pulse radiolysis of adrenodoxin reductase in the presence of 1 mM $NADP^+$ (A), NAD^+ (B), or NMA (C). It is noted that the reduction of the flavin in the presence of $NADP^+$ occurs more rapidly as compared with that in the presence of NAD^+ or NMA. Since [$NADP^+$]/[$NADP^+$—adrenodoxin reductase] is sufficiently high in this system, $NADP^•$ is formed preferentially in the bulk of the solution and not on the active site. Then, the $NADP^•$ reacts with $NADP^+$—adrenodoxin reductase complex to form the semiquinone of the enzyme. The rate constant of this process was independent of the concentration of the enzyme and is similar to that obtained in the presence of

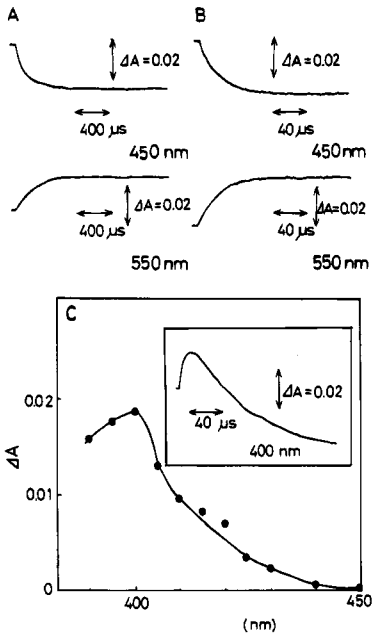


FIGURE 4: (A) Absorption changes after pulse radiolysis of adrenodoxin reductase measured at 450 and 550 nm. The reaction medium contained 42 μM enzyme, 0.1 M sodium formate, 10 mM phosphate buffer (pH 7.4), and N_2O. (B) Absorption changes after pulse radiolysis of $NADP^+$—adrenodoxin reductase measured at 450 and 550 nm. The reaction medium contained 42 μM enzyme, 42 μM $NADP^+$, 0.1 M sodium formate, 10 mM phosphate buffer (pH 7.4), and N_2O. (C) Kinetic difference spectrum at 5 μs after pulse radiolysis. The experimental condition is the same as in Figure 4B. The inset is absorption change after pulse radiolysis of $NADP^+$—adrenodoxin reductase measured at 400 nm.

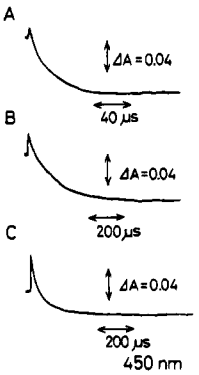


FIGURE 5: Absorption changes after pulse radiolysis of adrenodoxin reductase measured at 450 nm in the presence of (A) 1 mM $NADP^+$, (B) 1 mM NAD^+, or (C) 1 mM *N*-methylnicotinamide. The reaction medium contained 40 μM enzyme, 0.1 M *tert*-butyl alcohol, and 10 mM phosphate buffer (pH 7.4).

equimolar $NADP^+$. This suggests that $NADP^•$ transfers an electron to $NADP^+$ bound to the enzyme initially, and then the $NADP^•$ transfers an electron to the flavin by an intramolecular migration, as observed in the reaction of e_{aq}^- or CO_2^-. In this system, the rate-determining step is the electron transfer from bound $NADP^•$ to the flavin in the enzyme. A similar process was observed after pulse radiolysis of the $NADP^+$—adrenodoxin reductase complex in the presence of 1 mM NMA (data not shown). Under the condition, NMA radical reacted with the $NADP^+$ bound to the enzyme initially, and then an electron flowed to flavin. In the presence of NAD^+ or NMA, on the other hand, the rate constant was dependent on the concentration of the enzyme, indicating that $NAD^•$ or NMA radical directly reacts with the flavin of the enzyme.

The reaction of e_{aq}^- with adrenodoxin reductase was performed in the presence of adrenodoxin. The complex of adrenodoxin—adrenodoxin reductase was reduced little by e_{aq}^-, although the redox site of each protein could be reduced by e_{aq}^-. Upon the addition of 0.1 M NaCl to the system, reduction of both the flavin of adrenodoxin reductase and the iron—sulfur of adrenodoxin was observed. This indicates that two proteins are bound tightly to each other and mask their reduction sites completely.

DISCUSSION

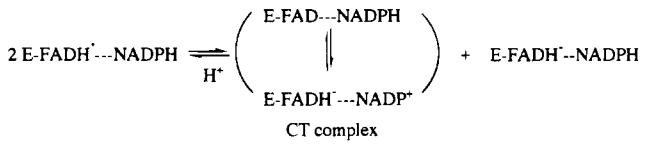
In a previous report, on the basis of the results of pulse radiolysis, we have classified flavoproteins as either e_{aq}^- reducible or unreducible (Kobayashi et al., 1988). The former class contains flavodoxin (Faraggi et al., 1979), ferredoxin $NADP^+$ reductase (Kobayashi et al., 1988), and cytochrome b_5 reductase (Kobayashi et al., 1988), which are functionally similar and serve as electron transports between these proteins and the corresponding oxidoreductase. In the latter class, with proteins such as D-amino acid oxidase (Kobayashi et al., 1983), glucose oxidase (Kobayashi et al., 1983), and lipoamide dehydrogenase (Elliot et al., 1980), e_{aq}^- did not react with the flavins, but with amino acid residues of the proteins instead. In the present experiment, the e_{aq}^- effectively reduced the flavin of adrenodoxin reductase. This result is consistent with the classification of flavoproteins based on the results of pulse radiolysis (Kobayashi et al., 1988). The flavin rings in the former class might be located in a similar environment. The edge of the dimethylbenzyl ring in both flavodoxin (Burnett et al., 1974) and ferredoxin $NADP^+$ reductase (Karplus et al., 1991) is exposed to the solvent, as has been suggested by X-ray analysis. The reaction with e_{aq}^- is assigned mainly to a direct reaction proceeding via the exposed flavin.

In the complex of $NADP^+$—adrenodoxin reductase, e_{aq}^- or CO_2^- did not react with the flavin, but with $NADP^+$ bound to the enzyme instead. This suggests that the nicotinamide moiety of $NADP^+$ is located near the surface of the protein molecule and masks the flavin completely. Such a result was not seen in $NAD(P)^+$ complexes in cytochrome b_5 reductase (Kobayashi et al., 1988), putidaredoxin reductase,[2] and ferredoxin $NADP^+$ reductase,[2] where e_{aq}^- reduced the flavins directly. This may reflect the difference in the relative arrangement of FAD and the nicotinamide moiety of $NAD(P)^+$ between adrenodoxin reductase (Miura & Ichikawa, 1994) and functionally related enzymes such as ferredoxin $NADP^+$ reductase and cytochrome b_5 reductase (Sem & Kasper, 1992). Indeed, comparison of the amino acid sequence of adrenodoxin reductase with those of several $NADP^+$-dependent enzymes shows no significant sequence similarity (Hanukoglu & Gutfinger, 1989).

The present experiment shows that e_{aq}^- failed to reduce the complex of adrenodoxin—adrenodoxin reductase, even though each member of the complex was readily reduced. This indicates that the entrances for an electron, where the redox centers are exposed to the solvent, are mutually covered upon formation of the complex. From the present data and noncompetitive binding of adrenodoxin and $NADP^+$ for adrenodoxin reductase (Miura & Ichikawa, 1994), the binding sites of the nicotinamide moiety of $NADP^+$, the flavin, and the iron—sulfur cluster may be located close to

[2] K. Kobayashi and S. Tagawa, unpublished results.

Scheme 2



each other in the ternary complex of $NADP^+$−adrenodoxin reductase−adrenodoxin. A similar structure has been proposed by a complex of ferredoxin−ferredoxin $NADP^+$ reductase (Karplus et al., 1991) and phthalate dioxygenase reductase (Correll et al., 1992), according to X-ray analysis. In phthalate dioxygenase, NADH, FMN, and the iron−sulfur cluster, bound to distinct domains, are brought together near a central cleft in the molecule.

When titrated with dithionite, adrenodoxin reductase accepts two electrons per molecule of FAD apparently without forming a semiquinone. From the present experiment, this can be explained in terms of the dismutation of the semiquinone, as shown in reaction 2. This process occurs through a direct collision between the proteins, since the reaction obeys second-order kinetics. A similar dismutation process was observed in the case of NAD^+-free cytochrome b_5 reductase (Kobayashi et al., 1988). Unlike adrenodoxin reductase, the semiquinone of b_5 reductase is stabilized by binding of NAD^+ (Iyanagi, 1977; Iyanagi et al., 1984). The NAD^+-bound semiquinone of b_5 reductase was obtained as a final product after pulse radiolysis (Kobayashi et al., 1988). Iyanagi et al. (1986) reported that the following disproportionate equilibrium between individual enzyme-bound flavin molecules of b_5 reductase is allowed in the presence of NAD^-.

$$2\text{E-FADH}^{\bullet} \underset{H^+}{\rightleftharpoons} \text{E-FAD} + \text{E-FADH}^-$$

In the case of adrenodoxin reductase, on the other hand, this equilibrium is considered far to the right even in the presence of $NADP^+$. The dismutation process was not affected by $NADP^+$. This difference may be explained by the redox potential of each state, where the redox potential of the E-FAD/E-FADH• is lower than that of the E-$FADH^-$/E-FADH•. On the other hand, Nonaka et al. (1986) reported that the semiquinone of adrenodoxin reductase was produced only with excess NADPH. In addition, the species between the charge-transfer complex and the semiquinone of adrenodoxin reductase were shown in an equilibrium by the use of resonance Raman and EPR spectra (Kitagawa et al., 1982). From this evidence, we propose that the following equilibrium in Scheme 2 exists in adrenodoxin reductase.

The equilibrium is allowed, when NADPH binds to the oxidized (charge-transfer complex), the semiquinone, and the fully reduced form of the reductase. The species (E-$FADH^-$−NADPH, E-FADH•−NADPH, and E-FAD−NADPH), which contain complexes with four, three, and two electrons, respectively, should play an important role in the switch from two- to one-electron transfer by adrenodoxin reductase. Nonaka et al. (1986) have discussed a similar mechanism for the formation of the semiquinone. This proposal is supported by the following preliminary experiment.[2] The e_{aq}^- was found to react with the charge-transfer complex of adrenodoxin reductase to form a stable product with an absorption maximum at 550 nm, characteristic of the semiquinone (data not shown). This product is considered to correspond to a NADPH-bound semiquinone. In addition, this result suggests that the flavin of the charge-transfer complex stays mainly in the oxidized state, which has been shown by the resonance Raman spectrum of the charge-transfer complex (Kitagawa et al., 1982). Further application of the pulse radiolysis technique to the oxidation−reduction states of the charge-transfer complex of adrenodoxin reductase is under way in this laboratory.

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