Adrenodoxin Reductase*  
PROPERTIES OF THE COMPLEXES OF REDUCED ENZYME WITH NADP+ AND NADPH

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J. DAVID LAMBETH* AND HENRY KAMIN

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Anaerobic reduction of the flavoprotein adrenodoxin reductase with NADPH yields a spectrum with long wavelength absorbance, 750 nm and higher. No EPR signal is observed. This spectrum is produced by titration of oxidized adrenodoxin reductase with NADPH, or of dithionite-reduced adrenodoxin reductase with NADP+. Both titrations yield a sharp endpoint at 1 NADP(H) added per flavin. Reduction with other reductants, including dithionite, excess NADH, and catalytic NADP* with an NADPH generating system, yields a typical fully reduced flavin spectrum, without long wavelength absorbance. The species formed on NADPH reduction appears to be a two-electron-containing complex, with a low dissociation constant, between reduced adrenodoxin reductase and NADP+, designated ARH$_2$-NADP$. Titration of dithionite-reduced adrenodoxin reductase with NADPH also produces a distinctive spectrum, with a sharp endpoint at 1 NADPH added per reduced flavin, indicating formation of a four-electron-containing complex between reduced adrenodoxin reductase and NADPH. Titration of adrenodoxin reductase with NADH, instead of NADPH, provides a curved titration plot rather than the sharp break seen with NADPH, and permits calculation of a potential for the AR/ARH$_2$ couple of $-0.291$ V, close to that of NAD(P)H ($-0.316$ V).

Oxidized adrenodoxin reductase binds NADP+ much more weakly ($K_{diss} = 1.4 \times 10^{-5}$ M) than does reduced adrenodoxin reductase, with a single binding site. The preferential binding of NADP+ to reduced enzyme permits prediction of a more positive oxidation-reduction potential of the flavoprotein in the presence of NADP+: a change of about $+0.1$ V has been demonstrated by titration with safranine T. From this alteration in potential, a $K_{diss}$ of $1.0 \times 10^{-4}$ M for binding of NADP+ to reduced adrenodoxin reductase is calculated. It is concluded that the strong binding of NADP+ to reduced adrenodoxin reductase provides the thermodynamic driving force for formation of a fully reduced flavoprotein form under conditions wherein incomplete reduction would otherwise be expected.

Stopped flow studies demonstrate that reduction of adrenodoxin reductase by equimolar NADPH to form the ARH$_2$-NADP$^+$ complex is first order ($k = 28$ s$^{-1}$). When a large excess of NADPH is used, a second apparently first order process is observed ($k = 4.25$ s$^{-1}$), which is interpreted as replacement of NADP$+$ for NADPH in the ARH$_2$-NADP$^+$ complex. Comparison of these rate constants to catalytic flavin turnover numbers for reduction of various oxidants by NADPH, suggests an ordered sequential mechanism in which reduction of oxidant is accomplished by the ARH$_2$-NADP$^+$ complex, followed by dissociation of NADP$^+$.

The absolute dependence of NADPH cytochrome c reduction on both adrenodoxin reductase and adrenodoxin is confirmed. Adrenodoxin reductase catalyzes 2,6'-dichlorophenolindophenol reduction by NADPH with two types of "intrinsic" rate constants: a relatively low one for adrenodoxin reductase itself, and a higher one for the previously reported 1:1 complex between adrenodoxin reductase and adrenodoxin.
transport chain, transferring reducing equivalents from NADPH to cytochrome P-450 (6, 9).

The flavoprotein can accept two electrons (2), and has been shown to form a 1:1 complex with the iron-sulfur protein, a one-electron acceptor (10, 11). Adrenodoxin reductase is specific for NADPH rather than for NADH, with apparent $K_m$ values for these pyridine nucleotides of 1.82 $\mu$M and 5.56 nM, respectively (2, 12).

Chu and Kimura (2) have described the spectrum of adrenodoxin reductase which had been anaerobically reduced with NADPH, and suggested that the low, broad absorbance extending from 505 to beyond 750 nm represents a "charge transfer complex" between NADP(H) and flavoprotein. Such a "complex" might be catalytically important and might contribute to the specificity for NADPH. This study was undertaken to determine the nature of the interaction between pyridine nucleotides and adrenodoxin reductase and to investigate the reduction of flavoprotein by this and other reductants.

We have found that NADP+ forms a low dissociation constant complex with reduced adrenodoxin reductase, but binds relatively weakly to oxidized adrenodoxin reductase; and that this preferential binding shifts the potential of the flavoprotein by almost 100 mV.

**EXPERIMENTAL PROCEDURE**

**Materials—**NADPH, NADP+, NADH, and 2'-monophosphoadenosine 5'-diphosphoribose were obtained from P-L Biochemicals; safranine T, from P & B Chemicals; glucose oxidase, type V, isocitrate, isocitrate dehydrogenase, nicotineamide, and Neurospora NADase, from Sigma; and DPIP from Mann, Inc. Adrenal glands were purchased from Swift and Co., Wilson, N.C.

**Methods—**Unless otherwise indicated all procedures were carried out in 10 mM KP, buffer, pH 7.5.

Adrenodoxin reductase was prepared from bovine adrenal cortex mitochondria according to a modification of the method of Omura et al. (6). Active fractions from the DEAE-cellulose chromatography step described in Ref. 6 were adsorbed onto a hydroxyapatite column (1.5 x 15 cm) and eluted with a gradient of KP, buffer, 300 ml, from 0.1 to 0.8 M. Fractions with a 272:450 nm absorbance ratio under 9 were pooled, concentrated, and chromatographed on a Sephadex G-75 column (1.5 x 25 cm). Resulting fractions had an absorbance ratio under 8.5, although this ratio increased to 9 or greater on storage and dialysis, probably due to loss of flavin. Fractions with final absorbance ratio greater than 11 were not used for experiments. About 10 mg of reductase were obtained per kg of adrenal cortex; yield could be increased to about 15 mg by including 1 to 2 mM neutralized EDTA in all buffers.

Adrenodoxin was prepared from the 60 to 80% ammonium sulfate fraction of the Omura et al. (6) procedure, and purified according to a modification of the method of Suhara et al. (13). The absorbance ratio, 414:272 nm, was 0.96.

Activities of adrenodoxin reductase were measured as follows. Reduction of DPIP by NADPH was monitored at 600 nm, with $e = 21,000$ $M^{-1} cm^{-1}$ (14). $K_{FeCN}$ reduction was monitored at 420 nm, with $e = 1,090$ $M^{-1} cm^{-1}$ (15). Cytochrome c reduction was monitored at 550 nm in the presence of excess adrenodoxin, using $e = 19,500$ $M^{-1} cm^{-1}$ (16). Only cytochrome c reduction showed an absolute requirement for adrenodoxin (2, 6). However, DPIP reduction was stimulated by adrenodoxin, as has been observed independently by Kimura and Cha (see Ref. 6), and by us.

Absorption of flavoprotein was obtained at 103 K and 173 K as previously described (17), using a Varian E9 HG spectrometer at a modulation frequency of 100 kHz, a modulation amplitude of 8 G, and a microwave power of 5 mW. Spectra were recorded with a 1.0-s time constant and a scan range of 3100 to 3400 G. Reactants were mixed anaerobically under a degassed argon atmosphere using an anaerobic EPR tube and mixing assembly (18). Final flavoprotein concentration was 50 to 90 $\mu$M (0 to 5 mg/ml).

Titrations were performed anaerobically under argon at room temperature according to the method and using the apparatus of Foust et al. (19). Trace oxygen was removed from argon by bubbling through three Erlenmeyer flasks, each containing 500 ml of Fieser’s solution (20). All solutions contained 10 mM glucose and 10 units/ml of glucose oxidase to insure continued anaerobiosis during the titration.

Binding of NADP+ to oxidized adrenodoxin reductase was determined by ultrafiltration (21), using a 50-ml ultrafiltration assembly with a PM-30 ultrafilter. A known amount of NADP+ was added to a known concentration of adrenodoxin reductase in 21 ml of 0.1 M KP, buffer within the assembly. Several fractions of filtrate, approximately 1 ml each, were collected at 2.5 p.s.i. with constant stirring. The concentration of free NADP+ was calculated from the absorbance at 258 nm of filtrate fractions. Apparent binding due to a small retardation of movement of NADP+ by the membrane was corrected for by repeating the experiment without flavoprotein, and subtracting apparent binding in the absence of protein from that in the presence of oxidation-reduction potentials were calculated from Equation 1, where $E_m$, is the midpoint potential at pH 7.5; $R$, the gas constant; $T$, the temperature in °K; $F$, Faraday’s constant; and $n$, the number of electrons required to reduce the appropriate species.

$$E_{m}(B) = E_{m}(A) + \frac{RT}{nF} \ln \frac{[A]}{[B]} - \frac{RT}{nF} \ln \frac{[B]}{[A]}$$

A and B refer to any two species under consideration, while the subscripts, ox and red, refer respectively to the oxidized and reduced forms of these species. The potential of the NADP+ complex of adrenodoxin reductase was determined by titrating anaerobically a mixture of the flavoprotein and safranine T with known quantities of NADPH. The concentration of oxidized safranine T was determined by its 520 nm extinction of 48,000 (10), and reduction by bleaching at this wavelength. Flavoprotein reduction was calculated by subtracting the amount of dye reduced from the number of electrons added as NADPH. The $E_{m}(B)$ of safranine T was determined similarly from the second phase of the titration following full reduction of flavoprotein, where species A is NADP(H) and B is safranine T. Assuming a potential of $-0.317$ V for NADPH, the calculated potential for safranine T was $-0.297$ V, in good agreement with the previously reported value at pH 7.0 of $-0.289$ V (10). In all calculations the oxidation-reduction potentials of NADP+/NADPH and NAD+/NADH are assumed equal (22, 23).

Stopped flow experiments were performed using a Durrum-Gibson stopped flow apparatus (24), with a Tektronix type 564 storage oscilloscope. Solutions were made anaerobic in tonometers, adapted to serve as stopped flow reservoirs (25), by flowing argon over the solutions for at least 30 min with frequent swirling, prior to loading in stopped flow syringes. Glucose and glucose oxidase were included as above. Absorbance calculations were made from photographs of the oscilloscope screen.

Photoreduction of flavoprotein was accomplished in 50 mM neutralized E111A using a fluorescent desk lamp as a light source (26).

**RESULTS**

**Absorption Spectra** of Reduced Adrenodoxin Reductase—We have confirmed the observation (2) that anaerobic reduction of adrenodoxin reductase with NADPH produces a spectrum with low, long wavelength absorbance (Fig. 1). The flavoprotein, reduced with 1.1 eq of NADPH, also shows residual absorbance at 450 and 377 nm, and isosbestic points for oxidized and NADPH-reduced enzyme at 507 and 382 nm. This spectrum does not resemble those of semiquinone forms hitherto described (27, 28), nor does it resemble a typical fully reduced epr spectrum (26, 27).

This calculation is valid for up to two electrons added because of the relatively positive potential of the ARH2+ NADP+ complex (see “Potentials”). A known quantity of NADP+ was added.

**APPENDIX**

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1. The abbreviations used are: DPIP, 2,6-dichlorophenolindophenol; AR, adrenodoxin reductase, oxidized form; ARH2 adrenodoxin reductase reduced form; ARH2+ NADP+, reduced adrenodoxin reductase-NADP+ complex.

2. We wish to thank Drs. Jean Johnson and K. V. Rajagopalan for performing the EPR experiments.
Reduction of flavoprotein with reducing agents alternate to equimolar NADPH yielded a spectrum typical of fully reduced flavin, without long wavelength absorbance or residual peaks at 450 or 377 nm (Fig. 2). We report for the enzyme following dithionite reduction (1). We confirm this report, and have also observed the fully reduced spectrum on photoreduction by light-EDTA. An isosbestic point for oxidized and reduced enzyme was seen at 336 nm. It is thus apparent that the enzyme form produced by reduction with an NADPH-generating system is different than that obtained by reduction with NADPH.

No spectra resembling those of known semiquinone forms were observed at intermediate titration points of the experiment described in Fig. 2, nor were they seen during photoreduction of flavoprotein, titration of photoreduced flavoprotein with ferricyanide, or anaerobic reduction with puridine nucleotides. Because reduction and reoxidation in these experiments was by small increments, and the solutions were allowed several minutes between additions and measurement, detectable spectra of semiquinone forms would not be expected if equilibrium between oxidized and reduced forms and semiquinone form (adrenodoxin reductase$_{ox}$ + adrenodoxin reductase$_{semiquinone}$) is far to the left, and if electron transfer between flavoprotein molecules occurs at an appreciable rate. A spectrum resembling that of neutral or "blue" semiquinone (26) was observed transiently, however, on reoxidation of NADPH-reduced flavoprotein by air (Fig. 3). The spectrum below 360 nm could not be evaluated because of the excess of NADPH.

EPR Spectra of Adrenodoxin Reductase—Neither oxidized adrenodoxin reductase, nor adrenodoxin reductase reduced aerobically with excess dithionite or anaerobically with stoichiometric NADPH, showed EPR signals at $g = 2.0$ or in the entire EPR scan range, suggesting that semiquinone forms were absent in these experiments. Because of the close correlation between EPR and visible spectra, no other concentrations of NADPH were tested, and the visible spectra were assumed to reflect the oxidation-reduction state of the flavoprotein.

Titrations of Adrenodoxin Reductase—Anaerobic titration of oxidized adrenodoxin reductase with NADPH (Fig. 4) showed decreasing absorbance at 450 nm and increasing absorbance at 700 nm, with a sharp break at 1 NADPH added per flavin, indicating a 1:1 stoichiometry for formation of a product containing two electrons.

Results of titration of dithionite-reduced adrenodoxin reductase with NADP$^+$ are shown in Fig. 5. Adrenodoxin reductase was first reduced with 1.1 eq of dithionite to produce the fully reduced spectrum (Fig. 5, curve 2). The reduced flavoprotein was then titrated with NADP$^+$, producing a spectrum identical to that obtained by reduction of oxidized flavoprotein with NADPH (Fig. 5, curve 3). The course of this titration (inset, Fig. 5) again indicated a 1:1 stoichiometry when observed at either 450 or 700 nm and again showed a sharp break point at 1 NADP$^+$ per FAD, indicating formation of a reduced species which had interacted with NADP$^+$. It is thus clear that this form can be produced by treating either oxidized enzyme with NADPH, or reduced enzyme with NADP$^+$, and that this species represents a two-electron-containing complex between...
Thus, there is no spectral evidence for complex formation between NAD+ and ARH.

The absorption spectrum of ARH·NADP+ complex—When 0.3 unit (0.2 mg) of NADase was added to adrenodoxin reductase previously reduced with a slight excess of dithionite. At increasing concentrations of NADH, the absorbance at 450 nm approaches the fully reduced absorbance. Thus, it appears that reduced adrenodoxin reductase can form a complex with NADPH as well as with NADP+.

Anaerobic titrations of oxidized adrenodoxin reductase with NADH yielded a curved titration plot (Fig. 7) rather than the sharp break seen on titration with NADPH. Data points for the titration are indicated by open circles. The theoretical titration plot, assuming complete reduction of flavoprotein (without detectable reversibility) by 2 reducing eq, is indicated by the dotted line, which was calculated on the basis of the 17% residual absorbance seen at 450 nm after full reduction by dithionite. At increasing concentrations of NADH, the absorbance at 450 nm approaches the fully reduced absorbance. Thus, there is no spectral evidence for complex formation between NAD+ and ARH.

Effect of Neurospora NADase on Absorption Spectrum of ARH·NADP+ Complex—When 0.3 unit (0.2 mg) of NADase was added to adrenodoxin reductase previously reduced with 1.2 eq of NADPH, a conversion from a spectrum like that of Fig. 8 to one resembling the fully reduced spectrum occurred over the course of about 4 hours. The NADase cleavage products (29) of NADP+ (nicotinamide and 2'-monophosphoadenosine 5'-diphosphoribose) were shown in a separate experiment to cause no spectral changes when added to dithionite-reduced flavoprotein.

Binding of NADP+ to Oxidized Adrenodoxin Reductase—Attempts at measuring NADP+ binding to oxidized adrenodoxin reductase by equilibrium dialysis were unsuccessful, since FAD was lost from the flavoprotein during the several days required to reach equilibrium. However, the ultratitration method described under "Experimental Procedure" allowed determination of a dissociation constant within several hours with no appreciable loss of FAD. Binding data were plotted according to the method of Scatchard et al. (30), and are consistent with a $K_d$ of 1.4 × 10⁻⁴ M (Fig. 8). Approximately one binding site per flavin is indicated. This is in reasonable agreement with a previously published $K_d$ for NADP+ of 5.32 × 10⁻⁴ M (2), and in good agreement with a $K_d$ for NADP+ of 2 × 10⁻⁴ M determined by us for ferricyanide reduction.

These experiments were carried out observing K$_3$FeCN$_4$ reduction as described under "Experimental Procedure," in the presence of various concentrations of NADP+. Kinetic analysis of Lineweaver-Burk plots showed competitive inhibition by NADP+ and allowed calculation of the $K_i$. The results of these experiments are unpublished.
Adrenodoxin Reductase: Interaction with Pyridine Nucleotides

Effect of NADP+ on Equilibrium Mixture of NADH/NAD+ and ARH/FAdrenodoxin Reductase—The titration of adrenodoxin reductase with NADH (Fig. 7) indicates that at 1 NADH added per flavin, there is only approximately a 70% reduction of the flavoprotein, i.e., an equilibrium mixture of oxidized and reduced forms. Fig. 9B shows the spectrum obtained by addition of equimolar NADH to flavoprotein, again showing no spectral evidence for an NAD(H)-flavoprotein complex, and indicating incomplete reduction of flavoprotein. The lower curve (Fig. 9D) indicates that the theoretical fully reduced spectrum calculated from the per cent residual absorbance after dithionite reduction. Addition of NADP+ to this equilibrium results in the spectrum of the ARH+/NADP+ species (Fig. 9C), a fully reduced species as shown by Figs. 4 and 5. Thus, the addition of an oxidized pyridine nucleotide causes, presumably by complex formation, the achievement of a fully reduced state.

Potential of Adrenodoxin Reductase—The curved titration plot obtained by reduction of adrenodoxin reductase by NADH (Fig. 7) indicates an equilibrium between oxidized and reduced forms of flavoprotein and NAD. From this equilibrium, the reduction potential for adrenodoxin reductase could be calculated from Equation 1, assuming an $E_m$ for NAD+/NADH of approximately $-0.316$ V. The solid curve shown in Fig. 7, is a theoretical line generated by assuming a difference in reduction potential between NADH and adrenodoxin reductase of 0.025 V, i.e., a reduction potential for AR/ARH$_2$ of approximately $-0.291$ V at pH 7.5. This is in reasonable agreement with a previously reported reduction potential of $-0.274$ V at pH 7.0, obtained by different methods.

The potential was also obtained for the adrenodoxin reductase-NADP+ complex by titration of flavoprotein with NADPH in the presence of approximately equimolar safranine T as described under “Experimental Procedure.” The titration observed at 520 nm was biphasic (Fig. 10), with a break at about 1 NADPH added per adrenodoxin reductase. Before this break there was only slight reduction of dye, indicating that flavin was being reduced to a large extent, followed by a more complete reduction of dye after the flavoprotein had been fully reduced. In two experiments, the potential of the flavoprotein in the presence of NADP+ was calculated from Equation 1 to be approximately $-0.198$ V (i.e., $-0.198$ V).

Rate of Formation of ARH$_2$/NADP+ Spectral Species—Stopped flow experiments in which various concentrations of NADPH were mixed anaerobically with 1.5 $\times$ $10^{-3}$ M adrenodoxin reductase, showed rapid formation of the ARH$_2$/NADP+ spectral species as judged by an approximately 50% decrease in absorbance at 450 nm and an increase at 700 nm, without evidence of formation of free reduced flavoprotein. At approximately equimolar concentrations of flavoprotein and NADPH reduction of adrenodoxin reductase followed a first order plot, and an apparent rate constant of $28$ s$^{-1}$ was calculated. At a 30-fold excess of NADPH, two distinct phases were apparent in

Calculations were based on absorbance changes at 450 nm. Changes at 700 nm were too small to permit accurate estimation of rates, but appeared to occur on the same time scale.
Adrenodoxin Reductase: Interaction with Pyridine Nucleotides

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**Discussion**

The interpretation of the spectrum shown in Fig. 1 as a complex between NADP+ and reduced adrenodoxin reductase (ARH₄) is supported by the following considerations.

**Table 1**

| Oxidant     | Flavin turnover | No. of experiments |
|-------------|-----------------|--------------------|
| DPIP*       | 1.77 ± 0.13     | 5                  |
| K_{FeCN}_{₄} | 10.96 ± 1.46    | 4                  |
| Cytochrome c⁺ | 4.48 ± 0.64    | 2                  |

*Initial rates of K_{FeCN}_{₄} and DPIP reduction were measured at 420 and 600 nm, respectively, using several concentrations of oxidant for each experiment. The numbers given are extrapolations to infinite concentrations of oxidant. Variation of NADPH above 10^-4 M caused no change in rate. Turnover is calculated on the basis of a two-electron process. The average turnover and range in several experiments is shown.

For each experiment, initial rate of cytochrome c reduction was measured at 550 nm in the presence of several concentrations of adrenodoxin in excess of adrenodoxin reductase. For each experiment, the number given represents the extrapolation to infinite adrenodoxin concentration. Variation of cytochrome c or NADPH above 10^-4 M caused no change in the rate.
The possibility that NAD$^+$ interacts with the flavoprotein without producing a spectral change cannot be rigorously ruled out. However, the lack of inhibition of enzymatic catalysis by NAD$^+$ (2) makes this possibility unlikely.

The observed spectrum was due to complex formation with pyridine nucleotide, and did not arise as an inevitable mechanistic consequence of reduction of the enzyme by NADPH. This was shown by the absence of this species when reduction was accomplished by a catalytic quantity of NADP$^+$ continually reduced by an NADPH-generating system.

The spectrum of the complex is converted to that of fully reduced free flavoprotein by Neurospora NADase which acts catalytically to cleave the nicotinamide ring from NADP$^+$. Thus, these studies clearly demonstrate a two-electron-containing complex (i.e., a "fully reduced" form) between pyridine nucleotide and flavoprotein.

The course of reduction of adrenodoxin reductase by NADPH and NADH is summarized in Fig. 12. Both pyridine nucleotides are assumed to have the same oxidation reduction potential, $-0.316$ V (22, 23). The oxidation-reduction potential of adrenodoxin reductase (2), in the absence of complex formation, should dictate an equilibrium between oxidized and reduced forms of flavoprotein and pyridine nucleotide as is seen during the NADH titration (Fig. 7). Such an equilibrium is shown in Equation 2.

$$NADH + AR = ARH_2 + NAD^+ \tag{2}$$

At 1 NADH added per FAD, there is only approximately a 70% reduction of flavoprotein. However, at 1 NADPH added per FAD, there is 100% reduction. Since NADP$^+$ binds only weakly to oxidized adrenodoxin reductase ($K_{diss} = 1.4 \times 10^{-5}$ M) (Fig. 8), this result is readily explained by the preferential complex formation of NADP$^+$ with reduced adrenodoxin reductase, as is shown in Equations 3, 4, and 5.

$$NADPH + AR = NADP^+ + ARH_2 \tag{3}$$

$$ARH_2 + NADP^+ = ARH_2-NADP^+ \tag{4}$$

Sum of Equations 3 and 4

$$NADPH + AR = ARH_2-NADP^+ \tag{5}$$

As with NADH, reduction by NADH, in the absence of complex formation, should result in an equilibrium between oxidized and reduced forms (Equation 3). However, NADP$^+$ binds tightly to reduced ARH$_2$, effectively removing free reduced flavoprotein from the equilibrium (Equation 4). The net result is the sum of Equations 3 and 4 (Equation 5), in which addition of NADPH stoichiometric to adrenodoxin reductase results in essentially 100% formation of a fully reduced species. Thus, complex formation with NADP$^+$ provides the thermodynamic driving force for full reduction of the flavoprotein under conditions wherein the reduction potentials of the flavin and the free pyridine nucleotide would otherwise dictate an equilibrium between oxidized and reduced forms.

Equations 2 and 4 predict, moreover, that two electrons from NADH may be used to form a fully reduced flavoprotein species if NADP$^+$ is added to bind ARH$_2$ and effectively remove it from the oxidation-reduction equilibrium. The net result is the sum of Equations 2 and 4 shown in Equation 6.

$$NADH + AR + NADP^+ = NAD^+ + ARH_2-NADP^+ \tag{6}$$

The results of such an experiment, in which NADP$^+$ was added to adrenodoxin reductase which had been incompletely reduced with 1 eq of NADH (Fig. 9), confirm this prediction, and result in the paradoxical, but predictable situation in which addition of an oxidized substrate drives a reaction toward complete reduction.

The preferential binding of NADP$^+$ to the reduced form of adrenodoxin reductase predicts that the oxidation-reduction potential of the flavoprotein will be made more positive in the presence of the ligand, NADP$^+$ (34). This prediction is verified in these studies. A difference of approximately 94 mV is seen comparing NADP$^+$-associated versus unassociated flavoprotein. Since the dissociation constant for binding of NADP$^+$ to oxidized adrenodoxin reductase has been established ($K_{diss} = 1.4 \times 10^{-1}$ M), the dissociation constant for binding of NADP$^+$ to reduced adrenodoxin reductase may be calculated from Equation 7.

$$K_d = K_{diss} \times 10^{-(2.303 \times \Delta E_m)} \tag{7}$$

where $K_d$ is the dissociation constant for binding of NADP$^+$ to reduced adrenodoxin reductase, $K_{diss}$ for that to oxidized adrenodoxin reductase, and $\Delta E_m$ the alteration in potential in the presence of NADP$^+$. The $K_d$ calculated from this equation is $1.0 \times 10^{-1}$ M.

This tight binding of pyridine nucleotide to the reduced flavoprotein occurs not only with NADP$^+$, but also with NADPH, as indicated by the sharpness of the endpoint when titrating reduced reductase with NADPH (Fig. 6). The binding site of reduced adrenodoxin reductase appears to have specificity for 2'-monophosphopyridine nucleotides. Since the oxidized enzyme has a lower affinity for this grouping, one may conjecture that a conformational change in the binding site accompanies reduction. The possible contribution to binding by charge transfer interaction in the ARH$_2$-NADP$^+$ complex cannot be evaluated from the present studies, since an accurate $K_{diss}$ for the ARH$_2$-NADP$^+$ complex could not be.
obtained for comparison with that of the ARH-NADP+ complex.

Stopped flow studies indicate that the formation of the ARH-NADP+ complex is rapid enough so that it could not be rate-limiting in the catalytic reduction of the three electron acceptors tested (Table I). Assuming complex formation as the rate-limiting step could account for a maximum flavin turnover of 28 to 30 s⁻¹, well above observed catalytic turnovers. There was no evidence for the appearance of free ARH during rapid reduction by NADPH, suggesting that the flavoprotein reacts with NADP+ to form the ARH-NADP+ complex as in Equation 5, rather than proceeding via the free reduced form.

The rate of DPIP reduction is sufficiently slow, however, so that DPIP either K,FeCN, or cytochrome c-adrenodoxin. The rate of Equation 5, rather than proceeding via the free reduced form. There was no evidence for the appearance of free ARH, during addition, the present data do not deal directly with the function of the iron-sulfur protein, adrenodoxin, which probably serves as the physiological electron acceptor.

function of the iron-sulfur protein, adrenodoxin, which probably serves as the physiological electron acceptor. Although supported by present data, remains tentative. In addition, the present data do not deal directly with the function of the iron-sulfur protein, adrenodoxin, which probably serves as the physiological electron acceptor.

The enzyme has not yet been observed in a single catalytic turnover event, so the mechanism presented in Equation 6, although supported by present data, remains tentative. In addition, the present data do not deal directly with the function of the iron-sulfur protein, adrenodoxin, which probably serves as the physiological electron acceptor.

The present studies have shown that NADP+ forms a complex with reduced adrenodoxin reductase and modifies its potential by about 0.1 V, so that when NADPH is used as reductant complete reduction of flavoprotein occurs. NADH, having the same oxidation-reduction potential as NADPH, produces only partial reduction of flavoprotein. Complex formation with 2-monophosphopyridine nucleotides could, therefore, represent a mechanism to insure efficient reduction of adrenodoxin reductase.

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