Adrenodoxin Reductase and Adrenodoxin

MECHANISMS OF REDUCTION OF FERRICYANIDE AND CYTOCHROME c*

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J. DAVID LAMBETH‡ AND HENRY KAMIN
From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Adrenodoxin reductase, the flavoprotein moiety of the adrenal cortex mitochondrial steroid hydroxylating system, participates in adrenodoxin-dependent cytochrome c and adrenodoxin-independent ferriyancide reduction, with NADPH as electron donor for both of these 1-electron reductions. For ferricyanide reduction, adrenodoxin reductase cycles between oxidized and 2-electron-reduced forms, reoxidation proceeding via the neutral flavin (FAD) semiquinone form (Fig. 9). Addition of adrenodoxin has no effect upon the kinetic parameters of flavoprotein-catalyzed ferriyancide reduction.

For cytochrome c reduction, the adrenodoxin reductase-adrenodoxin 1:1 complex has been shown to be the catalytically active species (Lambeth, J. D., McCaslin, D. R., and Kamin, H. (1976) J. Biol. Chem. 251, 7545-7550). Present studies, using stopped flow techniques, have shown that the 2-electron-reduced form of the complex (produced by reaction with 1 eq of NADPH) reacts rapidly with 1 eq of cytochrome c (k = 4.6 s⁻¹), but only slowly with a second cytochrome c (k = 0.1 to 0.3 s⁻¹). However, when a second NADPH is included, two more equivalents of cytochrome c are reduced rapidly. Thus, the adrenodoxin reductase-adrenodoxin complex appears to cycle between 1- and 3-electron-reduced states, via an intermediate 2-electron-containing form produced by reoxidation by cytochrome c (Fig. 10).

For ferricyanide reduction by adrenodoxin reductase, the fully reduced and semiquinone forms of flavin each transfer 1 electron at oxidation-reduction potentials which differ by approximately 130 mV. However, adrenodoxin in a complex with adrenodoxin reductase allows electrons of constant potential to be delivered from flavin to cytochrome c via the iron sulfur center.

We have previously shown that NADP⁺ remains bound to the 3- and 2-electron-reduced forms of the complex during catalysis, but can dissociate from the 1-electron-containing form, allowing reduction of the complex by the next molecule of NADPH.

NADP⁺ is a competitive inhibitor to NADPH in adrenodoxin reductase-catalyzed ferricyanide reduction (Kᵢ = 24 μM), but shows a "mixed" pattern of inhibition of the adrenodoxin-dependent cytochrome c reduction. Binding of NADP⁺ to a low affinity binding site on adrenodoxin reductase (Kᵢ = 200 μM), (a site different from the higher affinity site for reduction by NADPH) is shown spectrophotometrically to alter the interaction between adrenodoxin reductase and adrenodoxin. This can account for the non-competitive component of NADP⁺ inhibition observed for cytochrome c reduction.

Adrenodoxin reductase (EC 1.6.7.11), a single subunit, monoflavoprotein, contains flavin and adrenodoxin (adrenal ferrodoxin), an iron sulfur protein of the ferredoxin type, function as an electron transport chain from NADPH to cytochrome P-450 in the adrenal cortex mitochondrial steroid-hydroxylating systems (cholesterol side chain cleavage, 11β hydroxylation, and 18 hydroxylation) (1-5). The flavoprotein can accept 2 electrons from NADPH with the concomitant production of a long wavelength-absorbing (b) NADP⁺-reduced flavoprotein charge transfer complex (7, 8) and can transfer reducing equivalents from NADPH to hemoprotein, either the natural acceptor cytochrome P-450, or cytochrome c. Hemoprotein reduction requires the presence of adrenodoxin, a 1-electron carrier (1, 9-12).

Adrenodoxin and adrenodoxin reductase have been shown to form a tightly associated 1:1 complex when both components are in the oxidized form (12). We have previously shown this complex to be the catalytically active species for cytochrome c reduction and have demonstrated that at low ionic strengths the complex remains tightly associated, regardless of the observable oxidation states of either the flavoprotein or the iron-sulfur protein (11, 13). Thus, the complex should remain associated throughout a catalytic cycle.

Our previous studies used equilibrium methods to demonstrate and investigate 2- and 3-electron-reduced states of the flavoprotein:iron-sulfur protein complex. Reduction of the protein:protein complex with NADPH was shown to produce a 1:1:1 ternary complex of NADP⁺-adrenodoxin reduc-
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Adrenodoxin reductase, which contained 2 electrons assignable predominantly to the pyridine nucleotide-reduced flavoprotein charge transfer region (7, 11). However, such studies did not demonstrate a 1-electron-containing form of the protein-protein complex. Since cytochrome c, the electron acceptor in these studies, can accept only a single electron, such a 1-electron-containing species must be an obligatory product in the reoxidation of the 3-electron-containing complex by 1 eq of cytochrome c.

Present studies have utilized rapid mixing techniques to demonstrate a 1-electron-containing form of the enzyme and have investigated its interaction with NADP+. These studies have also shown that during catalysis of cytochrome c reoxidation, the adrenodoxin reductase-adrenodoxin cycle systems between 1- and 3-electron-reduced forms of the complex. A catalytic cycle for the flavoprotein-catalyzed ferricyanide reduction is also presented.

EXPERIMENTAL PROCEDURES

Materials—NADPH and NADP+ were obtained from P. I. Chemicals; K$_3$Fe(CN)$_6$ from Fisher; horse heart cytochrome c (type III), glucose oxidase (type V), isocitrate dehydrogenase, and isocitrate from Sigma. Adrenal glands were purchased from Swift and Co., Wilson, N. C.

Methods—All procedures were carried out in 10 mM KP$_2$ buffer, pH 7.5, containing 10$^-4$ M neutralized EDTA.

Adrenodoxin and adrenodoxin reductase were purified from beef adrenal cortex mitochondria by a modification of the method of Omura et al. (1), as described previously (7). A typical preparation began with 250 to 300 adrenal glands. Following sonication of the mitochondria, the supernatant was divided, with half used immediately to prepare adrenodoxin and adrenodoxin reductase, and half frozen for a later preparation. Both fresh and frozen supernatants gave satisfactory enzyme preparations.

The adrenodoxin reductase-adrenodoxin 1:1 complex was formed by mixing an equimolar amount of both proteins. An $E = 10,000$ cm$^-1$ was used for adrenodoxin reductase at 450 nm (8), and $E = 11,000$ M$^-1$ cm$^-1$ for adrenodoxin at 414 nm (14). The specificity and the absorbance ratios at 450 nm and 414 nm to 272 nm of the complex were stable to freezing and thawing followed by dialysis, whereas those of the individual proteins were diminished by the same treatment.

Adrenodoxin for stopped flow experiments was reduced catalytically, using 7.3 $\times 10^{-5}$ M adrenodoxin reductase plus either NADP+ in slight excess over adrenodoxin, or catalytic concentration of NADP+ (10$^-5$ M plus an NADP-generating system consisting of isocitrate dehydrogenase, 0.36 units/ml, and isocitrate (5 mM). The latter system was used in experiments (i.e. reaction of reduced adrenodoxin with oxidized adrenodoxin reductase) where it was desirable to exclude excess pyridine nucleotide. Solutions also contained 10 mM glucose and 10 units/ml of glucose oxidase to remove trace residual oxygen following anaerobiosis. Solutions were made anaerobic in 10-ml tubes (adapted to the Cary 14) equipped with side arms and stoppered with serum caps, by repeated evacuation and flushing with argon, as described previously (7). Following anaerobiosis, components were added, and reduction of adrenodoxin was followed in the Cary 14, measuring the absorbance change at 414 nm. Using the NADPH-generating system, reduction was complete within 30 min, and with NADPH alone, within 5 min. Reduction was sufficiently slow in both cases to be negligible when measuring the rapid reaction rates observed in stopped flow experiments. In addition, the NADPH-generating system served to keep the adrenodoxin fully reduced if slow oxygen leaks occurred during transfers or within the stopped flow apparatus.

Solutions were transferred anaerobically to a stopped flow loading apparatus consisting of a standard three-way stopcock with two side arms and stoppered with serum caps, by repeated evacuation and flushing with argon through a 20-gauge needle, respectively. Following flushing of the apparatus and syringe with anaerobic buffer, the needle was inserted into the side arm of the tube containing the reduced adrenodoxin, and the contents were withdrawn into the stopped flow reservoir syringe. A second needle attached to the argon gas line (1 atm) was inserted in the tube to allow contents to be loaded without creating a vacuum. Control experiments described under "Results" confirmed that the adrenodoxin had remained reduced during transfer and throughout the series of experiments. Other reagents were degassed and transferred in the same manner.

Stopped flow experiments utilized a Durrum-Gibson stopped flow apparatus (15), with a Tektronix type 564 storage oscilloscope. For anaerobic experiments, the stopped flow apparatus was washed prior to use first with argon-bubbled buffer, then with the same buffer containing 10 mM glucose plus 10 units/ml of glucose oxidase. Absorbance calculations were made from photographs of the oscilloscope screen.

Oxidation-reduction potentials were calculated from the Nernst equation, as described previously (7, 11).

Initial rates of cytochrome c and K$_3$Fe(CN)$_6$ reduction were measured at 550 nm and 420 nm, respectively, using $e = 19,100$ M$^-1$ cm$^-1$ (16), and $e = 1,920$ M$^-1$ cm$^-1$ (17).

Spectra were recorded using a Cary 14 recording spectrophotometer.

RESULTS

Semiquinone Form of Adrenodoxin Reductase—We have previously observed a spectrum resembling that of the "blue" or neutral form of flavin semiquinone, during air reoxidation of adrenodoxin reductase which had been reduced with an excess of NADPH (7). To demonstrate that this spectrum did indeed reflect a 1-electron-containing species, we mixed adrenodoxin reductase with equimolar (NADPH plus K$_3$Fe(CN)$_6$) in the stopped flow apparatus. Since NADPH donates two reducing equivalents to the flavoprotein, and K$_3$Fe(CN)$_6$ equimolar to flavoprotein should accept a single electron from many of the reduced flavoprotein molecules, many of the flavins should end up in the 1-electron-containing form. Fig. 1, dotted line, shows the spectrum constructed from six stopped flow experiments at various wavelengths, plotting final absorbance at the end of 100 ms as a function of wavelength. A spectrum typical of neutral semiquinones is produced and may be compared to the spectrum of oxidized flavoprotein in this region (Fig. 1, solid line). Residual absorbance at 700 nm suggests that in addition to the 1-electron-containing form, a significant amount of 2-electron-reduced flavoprotein in charge-transfer association with NADP+ (7) remains. Since absorbance of this complex is flat from 500 to 700 nm, its presence does not interfere with the observation of the characteristic spectrum of the neutral flavin semiquinone.

In a control nonenzymatic experiment, K$_3$Fe(CN)$_6$, at the same concentrations as in this experiment, caused less than a 5% oxidation of NADPH as judged by decreased 340 nm absorbance over a 2-h period.
One Electron-containing Form of Adrenodoxin Reductase. Adrenodoxin Complex, and Oxidation-Reduction Potential of Flavin Semiquinone. —Since adrenodoxin reductase forms a low dissociation constant 1:1 complex with adrenodoxin \( K_{diss} = 10^{-9} \text{ M} \) (11, 12), a useful method for quantitative production of a 1-electron-containing form of the protein-protein complex should be mixing of reduced adrenodoxin with equimolar oxidized adrenodoxin reductase. Table I shows the 600 nm absorbance produced at the end of the reaction (100 ms after anaerobic rapid mixing of the components), and compares this to the absorbance of possible products of the reaction. Reduced adrenodoxin was produced as described under "Experimental Procedures," and remained reduced throughout the series of experiments as demonstrated by Control Experiments 1, 3, and 4 in Table I; these experiments were performed in the indicated order. Experiment 2 of Table I shows that reaction of reduced adrenodoxin with oxidized adrenodoxin reductase results in increased absorbance at 600 nm, an increase above that possible for oxidation of adrenodoxin alone. The reaction was completed within the dead time of the stopped flow instrument (3 ms) and the absorbance was stable for at least several seconds. No EPR signal (either at \( g = 2.00 \) or at \( g = 1.94 \)) was observed for this species. However, 30 s or more were necessary to mix and freeze the components in an anaerobic EPR assembly, and significant disproportion of electrons may have occurred during this time. This possibility was not tested experimentally (e.g. stopped flow or absorbance spectra) since any observed absorbance changes during relatively long time periods would be complicated by the slow reduction of the system by the NADPH generating system (see "Experimental Procedures").

The increased absorbance at 600 nm is attributed to oxidation of reduced adrenodoxin by flavoprotein (\( \epsilon (\text{adrenodoxin})_{600} = +1100 \text{ M}^{-1} \text{ cm}^{-1} \)), with concomitant formation of neutral semiquinone. An accurate extinction coefficient for the latter species has not been determined, and the oxidation-reduction potentials of adrenodoxin reductase, adrenodoxin (Ref. 11 and present studies) predict that intermolecular disproportionation, with ultimate formation of fully reduced flavin, is energetically favored. This prediction is strengthened by the observation that, at "half-reduction" of adrenodoxin reductase produced by either limiting NADPH or light-EDTA (7), only a mixture of fully oxidized and fully reduced forms were observed, with no detectable contribution from semiquinone.

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### Table I

| Experiment | Observation wavelength | Possible reaction product(s) | Absorbance predicted for corresponding product | Absorbance observed |
|------------|------------------------|------------------------------|-----------------------------------------------|---------------------|
| 1. Adrenodoxin (reduced) (13.3 \( \mu \text{M} \)) plus Buffer (anaerobic) | 600 | Adrenodoxin (reduced) | 0.026 | 0.027 |
| 2. Adrenodoxin (reduced) (13.3 \( \mu \text{M} \)) plus Adrenodoxin reductase (oxidized) (13.3 \( \mu \text{M} \)) | 600 | Adrenodoxin (reduced) + Adrenodoxin reductase (oxidized) | 0.043 | 0.036 |
| 3. Adrenodoxin (reduced) (13.3 \( \mu \text{M} \)) plus Cytochrome c (oxidized) (12.0 \( \mu \text{M} \)) | 550 | Adrenodoxin (oxidized) + cytochrome c (reduced) | 0.377 | 0.372 |
| 4. Buffer plus Cytochrome c (oxidized) (12.0 \( \mu \text{M} \)) | 550 | Cytochrome c (oxidized) | 0.066 | 0.111 |

* This calculation is based on an assumed extinction coefficient for neutral flavin semiquinone of 4000 \( \text{ M}^{-1} \text{ cm}^{-1} \).
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cytochrome c reduction. This is in accord with the catalytic studies by Chu and Kimura, and others (12, 19, 20), who suggest that the low level of NADPH oxidase activity of adrenodoxin plus adrenodoxin reductase, seen in the absence of electron acceptors, produces superoxide, but find that superoxide dismutase has only a small effect on the rate of cytochrome c reduction by this enzymatic system.

Reduced adrenodoxin is also shown (Fig. 2) to react with K₄Fe(CN)₆ another single electron acceptor. The pseudo-first order rates of reoxidation show no evidence of saturation behavior on increasing K₄Fe(CN)₆ concentrations up to ~4 × 10⁻⁸ M, indicating that no kinetically significant complex of adrenodoxin and K₄Fe(CN)₆ participates in this concentration range. Assuming second order kinetics, a rate constant of 1.8 × 10⁻⁹ s⁻¹ M⁻² is calculated.

Binding of NADP⁺ to 1-Electron-Containing Form of Adrenodoxin Reductase·Adrenodoxin Complex—NADP⁺ addition caused an increase in the 600 nm absorbance of the 1-electron-containing form of the adrenodoxin reductase·adrenodoxin complex. Fig. 3 shows the increase in 600 nm absorbance observed on stopped flow mixing of adrenodoxin plus adrenodoxin reductase, when the indicated concentration of NADP⁺ is included in the adrenodoxin reductase mixing syringe. An apparent "saturation" of this absorbance change is observed at high NADP⁺ concentration. By assuming a single binding site in this concentration range for pyridine nucleotide (7), a dissociation constant for NADP⁺ binding to the 1-electron-containing complex could be calculated. The value determined from the concentrations of each species at 25 µM NADP⁺ added was 25 µM. The Δε for this binding process, used to calculate the concentration of the complex with bound NADP⁺, was determined from the absorbance change at 600 nm extrapolated to infinite NADP⁺ concentration. The dissociation constant obtained is similar to that for binding of NADP⁺ to oxidized adrenodoxin reductase (in the absence of adrenodoxin), 14 µM (Ref. 7 and present studies), but is well above that for binding to the 2-electron-reduced adrenodoxin reductase, 1 × 10⁻⁸ M (7). NADP⁺ is also bound with a low Kₐ₂₃ not determined).

NADPH binding to oxidized adrenodoxin reductase or adrenodoxin reductase·adrenodoxin complex has not been measured directly, since reduction of the flavoprotein by NADPH occurs rapidly, and forms the low dissociation constant-reduced flavoprotein·NADPH complex. However, Kₐ values for NADPH, determined from catalytic studies with either adrenodoxin-dependent or independent reactions, are both 2 to 3 µM (Ref. 8 and present studies). Thus, adrenodoxin binding to adrenodoxin reductase does not appear to exert a major effect on binding of pyridine nucleotides. Small changes are not ruled out, however, and might be expected to be reflected in the effect of adrenodoxin on the rate of reduction of adrenodoxin reductase by NADPH.

Effect of Adrenodoxin on Rate of Reduction of Adrenodoxin Reductase by NADPH—Table II summarizes the effect of adrenodoxin on the rate of reduction of adrenodoxin reductase.

We have shown previously (7) that the product of the reduction of adrenodoxin reductase by equimolar NADPH, either in the presence or absence of adrenodoxin, is the NADP⁺-reduced flavoprotein charge transfer complex. As indicated in Table II, the first order rate constant for reduction of adrenodoxin reductase by NADPH is decreased from 28 s⁻¹ to 18 s⁻¹ in the presence of adrenodoxin. In both cases the total absorbance change was approximately 50% that of oxidized flavin, a value consistent with formation of the NADP⁺-reduced flavoprotein complex. A series of plots of the rate data from Experiment 3, Table II, in the form of A/t versus t, A/t² versus t, etc., according to the treatment of Darvey et al. (21) yields a positive slope in the first plot, with a negative slope in subsequent plots, indicating the formation of one intermediate prior to formation of the charge transfer complex. Such an intermediate has been suggested by us previously (7) to be the NADPH-oxidized adrenodoxin reductase complex.

In experiments with catalytic adrenodoxin reductase, we and others have shown that the rate of NADPH-dichlorodihydroxy-phenol oxidation-reduction is stimulated by adrenodoxin (11–13) and that this stimulation reflects catalysis of reduction by the adrenodoxin reductase·adrenodoxin complex instead of by the flavoprotein alone (11). Present studies reveal that the rate stimulation by adrenodoxin is not due to an increased rate of flavoprotein reduction, since adrenodoxin decreases rather than increases this rate. The rate stimulation must, therefore, be on the reoxidative side of the reaction.

Catalysis of NADPH·cytochrome c Oxidation-Reduction by Adrenodoxin Reductase·Adrenodoxin Complex—Cytochrome c reduction was observed at 550 nm after stopped flow mixing of adrenodoxin reductase·adrenodoxin with various ratios of NADPH and cytochrome c (Table III). Reduction of the com-

![Fig. 2 (left). Rate of reaction of reduced adrenodoxin with K₄Fe(CN)₆. Reduced adrenodoxin (14 µM) in reaction syringe 1, prepared as described under "Methods," was reacted with the four indicated concentrations of K₄Fe(CN)₆, in reaction syringe 2 of the stopped flow spectrophotometer, and the apparent first order rate constants for reoxidation of adrenodoxin were determined from log plots of the rate of absorbance change at 500 nm. Final concentrations after mixing are one-half the indicated values.

![Fig. 3 (right). Binding of NADP⁺ to the 1-electron-containing form of the adrenodoxin reductase·adrenodoxin complex. Reduced adrenodoxin (12.5 µM) in syringe 1 of the stopped flow was mixed anaerobically with equimolar oxidized adrenodoxin reductase in the presence of the indicated concentrations of NADP⁺. The absorbance (600 nm) is the difference between the absorbance in the presence and absence of NADP⁺.

| Experiment | Rate (s⁻¹) | Reference |
|------------|------------|-----------|
| 1. Adrenodoxin reductase (15.0 µM) NADPH (15.0 µM) | 28 | (G) |
| 2. Adrenodoxin reductase (15.0 µM) NADPH (450.0 µM) | 30 | (6) |
| 3. Adrenodoxin reductase (8.5 µM) + Adrenodoxin (8.5 µM) NADPH (8.5 µM) | 18 This work |
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Catalysis of cytochrome c reduction by adrenodoxin reductase·adrenodoxin complex

| Experiments | First phase | Second phase |
|-------------|-------------|--------------|
|             | Rate [Cytochrome c reduced] | Rate [Cytochrome c reduced] |
|             | [AR·ADX]    | [AR·ADX]     |
| 1. Adrenodoxin reductase·adrenodoxin (8.5 μM) NADPH (8.5 μM) + cytochrome c (8.5 μM) | 4.6 | 0.78 |
| 2. Adrenodoxin reductase·adrenodoxin (8.5 μM) NADPH (8.5 μM) + cytochrome c (17.0 μM) | 4.6 | 0.93 |
| 3. Adrenodoxin reductase·adrenodoxin (8.5 μM) NADPH (17.0 μM) + cytochrome c (17.0 μM) | 5.12 | 2.03 |
| 4. Adrenodoxin reductase·adrenodoxin (4.25 μM) NADPH (8.5 μM) + cytochrome c (17.0 μM) | 2.27 | 2.06 |

* A 5 extinction coefficient at 550 nm of 17,500 instead of 19,100 M⁻¹ cm⁻¹ was used to calculate the cytochrome c reduction in the first phase, since a loss of the 550 nm absorbance of the NADP⁺·AR·ADX charge transfer species should occur following the first 1-electron oxidation of the complex by cytochrome c. The 1-electron flavin semiquinone-reduced adrenodoxin equilibrium (Equation 1) produced by this reaction would not alter the extinction coefficient significantly, since at this wavelength calculations indicate that the decreased absorbance due to adrenodoxin reduction, and the increased absorbance due to semiquinone formation would be approximately equal.

When a second cytochrome c was included in the reaction (Table III, Experiment 2), a second, very slow phase of cytochrome c reduction was observed. The two phases are shown by the open circles in Fig. 4. The amounts of cytochrome c reduced in rapid and slow phases were approximately equal, and the total amount reduced was 93% of the available cytochrome c (see Table III). Reduction of the second cytochrome c by the 1-electron-containing form of the complex is far too slow to account for the observed rate of turnover in catalytic experiments. Therefore, a mechanism in which adrenodoxin reductase-adrenodoxin is first reduced by an NADPH and then reoxidized by two successive reductions of cytochrome c (i.e. cycling of the complex between fully oxidized and 2-electron-reduced), is ruled out.

However, when a second equivalent of NADPII is included along with the second cytochrome c (Table III, Experiment 3) both equivalents of cytochrome c are reduced at a rate approximately equal to the observed turnover rate in catalytic experiments. No slower phase of reduction can be discerned. Thus, two equivalents of NADPH were required to reduce two cytochrome cs at a rapid rate. The most likely mechanism for this phenomenon is that the second NADPH is further reducing the AR·ADX complex to a 3-electron-reduced form, AR·ADX⁴, which then reacts rapidly with the second cytochrome c.

Since the product of the latter reaction would again be the 2-electron-reduced form, such a mechanism predicts that if four cytochrome cs and two NADPHs per adrenodoxin reductase·adrenodoxin complex are reacted, three of the four cytochrome cs should be reduced rapidly, while the fourth should be reduced slowly. The results of such an experiment (Table III, Experiment 4) confirm this prediction; the closed circles in Fig. 4 show that 75% of the 550 nm absorbance change occurs in the rapid phase. The total absorbance change represents 93% of the theoretically reducible cytochrome c. Thus, in turnover, the adrenodoxin reductase·adrenodoxin complex catal). This experiment was suggested by Dr. K. V. Rajagopalan of Duke University and was performed with his collaboration.
must be cycling between the 1-electron-reduced and 3-electron-reduced forms.

**Catalytic Properties of Adrenodoxin Reductase and Adrenodoxin** — Adrenodoxin reductase catalyzes the NADPH-dependent reduction of $K_\text{Fe(CN)}_6^\text{3-}$. The double reciprocal plot of ferricyanide reductase activity versus NADPH concentration at four different NADP$^+$ concentrations is shown in Fig. 5. The data indicate that the inhibition by NADP$^+$ is competitive with NADPH. Although a $K_m$ for NADPH could not be determined directly from the line in Fig. 5 at zero NADP$^+$, a plot of the apparent $K_m$ values ($K_{\text{observed}}$) taken from the x-intercepts at various NADP$^+$ concentrations in Fig. 5, versus NADP$^+$ concentration allowed determination of the $K_m$ (see Fig. 6). A $K_m$ for NADPH of 2.6 $\mu$M and a $K_f$ for NADP$^+$ of 24.0 $\mu$M are determined from the x- and y-intercepts of this plot according to the following equation:

$$K_{\text{observed}} - K_m (1 + (I/K_f))$$

(2)

Adrenodoxin stoichiometric to adrenodoxin reductase has no effect on either the rate of ferricyanide reduction or the pattern of inhibition by NADP$^+$ for this reaction.

The steady state kinetics of cytochrome $c$ reduction by adrenodoxin reductase plus adrenodoxin, have been investigated previously (12); a $K_m$ of 1.8 $\mu$M for NADPH was determined, and a double reciprocal plot of velocity of cytochrome $c$ reduction versus NADPH concentration, with varied NADP$^+$ concentration, showed a pattern of “mixed” (12) inhibition by NADP$^+$. We have confirmed this result; the lines at zero NADP$^+$ and at four different NADP$^+$ concentrations intersect to the left of the y-axis and above the x-axis. A $K_m$ for NADPH of 2.0 $\mu$M was determined, in good agreement with the results of Chu and Kimura.

**Binding of NADP$^+$ to Adrenodoxin Reductase-Adrenodoxin Complex** — The observation of “mixed” inhibition by NADP$^+$ in cytochrome $c$ reduction and “pure competitive” inhibition in ferricyanide reduction suggested to us that the difference between the two patterns could reflect the requirement for adrenodoxin in the cytochrome $c$ but not the ferricyanide reaction. We therefore sought possible optical changes which could reflect an effect of NADP$^+$ on adrenodoxin-adrenodoxin reductase interactions.

NADP$^+$ binding has been shown to induce a perturbation of the spectrum of adrenodoxin reductase (18). The difference spectrum produced is shown in Fig. 7, following addition of 0.45 (Fig. 7A, dashed line) or 0.90 (Fig. 7A, solid line) equivalents of NADP$^+$ to the flavoprotein-iron-sulfur protein complex. An isosbestic point is seen at 482 nm, with a peak at 498 nm and decreased absorbance at wavelengths below 482 nm. However, on addition of NADP$^+$ above 1.4 eq (Fig. 7A, other curves), isosbesticity is lost, and a new absorbance peak is seen at 392 nm. These data demonstrate sequential formation of at least two different species on titration of the protein-protein complex with NADP$^+$, suggesting at least two different binding sites (with different binding constants) for pyridine nucleotides. The difference spectrum due to binding to the high affinity binding site is seen on addition of a less than stoichiometric amount of NADP$^+$ to the protein-protein complex (Fig. 7B, dashed line). The difference spectrum produced on binding to the low affinity site was calculated by subtraction of the difference spectrum at 4.55 eq from that at 13.6 eq of NADP$^+$ (Fig. 7B, solid line), and shows a trough at 496 nm, and an isosbestic point at 487 nm, with increased absorbance below 487 nm.

Both types of NADP$^+$-induced absorbance perturbations oc-
cur whether or not adrenodoxin is present, indicating both binding sites to be on the flavoprotein. Binding to the high affinity site produces no long wavelength absorbance changes (beyond 540 nm) either in the presence or absence of adrenodoxin. However, binding of NADP to the low affinity binding site produces longer wavelength absorbance changes when adrenodoxin is present, but not when it is absent. Because adrenodoxin, but not adrenodoxin reductase absorbs in this region, these data indicate that the binding of NADP to the low affinity site alters the flavoprotein-iron sulfur protein interaction in some way. This alteration does not involve dissociation of the complex, since the characteristic difference spectrum for dissociation of this complex (10) is not produced. Dissociation of the complex would be expected to produce a decrease rather than the observed increase in the absorbance beyond 540 nm. In addition, this absorbance cannot be due to flavin semiquinone or charge transfer complex, since all components are fully oxidized.

The concentration of adrenodoxin-adrenodoxin reductase with NADP bound to the high and low affinity sites may be determined from the absorbance changes at 487 nm and 482 nm, respectively, with extinction coefficients for each of these species determined by extrapolation of the absorbance change at each wavelength to infinite NADP concentration. The titration of the complex with NADP followed at these wavelengths and at 560 nm is shown in Fig. 8. Two clearly distinguishable binding processes were seen to be associated with 487 nm and 482 nm absorbance changes. Long wavelength absorbance changes (560 nm) are shown in Fig. 8 to have the same NADP concentration dependence as the 482 nm changes. No binding to the low affinity site is seen until a greater than stoichiometric amount of NADP is added, as indicated by the lag period preceding absorbance changes at 560 and 482 nm. Assuming that binding of one NADP to each of the two binding sites produces each of the observed difference absorption spectra, the concentration of unbound NADP at any NADP enzyme complex ratio can be calculated, and from this, dissociation constants for binding of NADP to the high and low affinity binding sites on the flavoprotein. These constants were calculated from the data obtained at 1 and 10 eq of NADP per mol of enzyme complex; the values were 13 and 200 μM, respectively. We had previously demonstrated by ultrafiltration binding studies (7) a high affinity site for binding of one NADP to adrenodoxin reductase, (K = 14 μM), in agreement with present studies. The earlier studies could not have detected the low affinity binding site, because of the limited concentration range studied.

**DISCUSSION**

Catalysis of NADPH-KFe(CN) oxidation-reduction by adrenodoxin reductase is proposed to proceed via the neutral flavin semiquinone intermediate as demonstrated by Fig. 1, according to the mechanism in Fig. 9. The product of NADPH reduction of adrenodoxin reductase, formed rapidly, is the charge transfer complex, in which the NADP is tightly bound (K = 3 x 10^-8 M) to the reduced flavoprotein (7). Dissociation of NADP then occurs at some step following the reduction of the first ferricyanide. The sequence of dissociation of NADP versus oxidation by a second KFe(CN) is undefined in this proposed mechanism, since rates for these processes are unknown. The catalytic cycle of adrenodoxin reductase with ferricyanide (Fig. 9), is reminiscent of the cytochrome b reductase system (22), in which a one-FAD enzyme, probably in charge transfer complex with NADH, reduces 2 mol of a 1-electron acceptor (cytochrome b5 or ferricyanide) in two successive steps, using both the fully reduced flavin and the semiquinone as reductants.

Competitive inhibition of NADPH binding by NADP is permitted by this proposed mechanism. The observed K of 23 μM compares reasonably well with the observed K of 13 to 14 μM described in the present and previous (7) studies. Neither the rate of KFe(CN) reduction, nor the pattern of inhibition by NADP is affected by the presence of adrenodoxin stoichiometric to adrenodoxin reductase, suggesting that when present, the iron-sulfur center does not participate in the reduction of KFe(CN)6. This suggestion is further supported by the second order rate constant of 1.8 x 10^-6 M^-1 s^-1 for reduction of KFe(CN)6 by reduced adrenodoxin. At catalytic concentrations of adrenodoxin, this could account for a rate of ferricyanide reduction on the order of only 0.01 s^-1. Thus, adrenodoxin does not alter the mechanism of NADPH-ferricyanide oxidation reduction by adrenodoxin reductase and should not participate in this reaction.

Catalysis of NADPH-cytochrome c reduction, however, requires the presence of both adrenodoxin reductase and adrenodoxin, in a 1:1 ratio (11). The proposed mechanism for the cytochrome c reduction activity of the adrenodoxin reductase-adrenodoxin complex is summarized in Fig. 10. The complex first reacts with NADPH with an apparent first order rate constant of 18 s^-1, to produce the NADP-AR-ADX species, a form which contains 2 electrons in a reduced flavin-NADP charge transfer complex of low dissociation constant (1 x 10^-8 M) (7). Analysis of the kinetics of reduction indicates a single intermediate in this reaction, which we have previously sug-

![Fig. 8](image-url)  
**Fig. 8.** Titration with NADP followed at 482, 487, and 560 nm. Absorbance values are corrected for dilution. Initial concentration of adrenodoxin reductase-adrenodoxin complex (AR-ADX) was 22.0 μM.

![Fig. 9](image-url)  
**Fig. 9.** Mechanism of NADPH-KFe(CN)6 reduction catalyzed by adrenodoxin reductase (AR).
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Fig. 10. Mechanism of NADPH-cytochrome c reduction, catalyzed by the adrenodoxin reductase-adrenodoxin 1:1 complex (AR-ADX).

suggests to be the reduced pyridine nucleotide-oxidized flavoprotein complex, prior to reduction of the flavin (7). These two reactions are shown in Fig. 10, A and B.

Adrenodoxin, the natural electron acceptor for adrenodoxin reductase (1-3), is an electron acceptor (14) and is tightly bound in a 1:1 complex to adrenodoxin reductase (12). We have previously shown that the complex remains tightly associated, regardless of the observable oxidation states of either protein (11). Transfer of 1 electron from the fully reduced flavin center in adrenodoxin reductase to the iron-sulfur center in adrenodoxin would produce the species AR-ADX (Fig. 10, Reaction C). Although such a species has not been observed in equilibrium or rapid reaction studies, its existence is supported by the following considerations. First, adrenodoxin reductase alone exhibits no cytochrome c reductase activity, and carries out this reaction only in the presence of adrenodoxin (1, 9-12). Second, reduced adrenodoxin reacts rapidly (k > 300 s\(^{-1}\)) to reduce cytochrome c, in an oxygen independent reaction (present studies). Third, reaction of NADPH with equimolar enzyme complex, in the presence of an equivalent of cytochrome c, is but slowly reactive (k = 4.6 s\(^{-1}\)) reduction of the cytochrome c (see Table III). Thus, we conclude that in order for the 2-electron-reduced complex to reduce cytochrome c, one of the electrons must reside in the iron-sulfur center. In addition, thermodynamic considerations support the existence of such a species. The free energy change (\(\Delta G\)) for Reaction C in Fig. 10 may be calculated from the following scheme.

\[
\text{AR-ADX} + e^- \rightarrow \text{AR-ADX} ^+ \quad \Delta G_1
\]

\[
\text{AR-ADX} \rightarrow \text{AR-ADX} ^+ 1e^- \quad \Delta G_2
\]

where \(\Delta G_1 = \Delta G_1 - \Delta G_2\). \(\Delta G_1\) is calculated from the equation \(\Delta G_1 = -RT \ln K_1\), and \(\Delta G_2\) from the equation \(\Delta G_2 = nFE_{\text{mq}}\), where the equilibrium constant \(K_1\), and the midpoint potential \(E_{\text{mq}}\) are determined from present studies. \(\Delta G_1\) may be calculated similarly from the flavin semiquinone/fully reduced midpoint potential, which is in turn calculated from \(E_{\text{mq}}\) and the known midpoint potential for the flavin oxidized/fully reduced couple (11, 13). A value of about 2400 calories is calculated for the energy required to transfer an electron from AR-ADX to form AR-ADX. In terms of an equilibrium constant, only approximately one out of sixty of the 2-electron-reduced species would be in the latter form. This finding explains the inability to observe this species in equilibrium or rapid reaction studies (11).

The free energy changes for reduction of the adrenodoxin reductase-adrenodoxin complex by NADPH, followed by reoxidation by two subsequent reactions with 2 eq of cytochrome c, are summarized in Fig. 11, using the formulation introduced by Weber (23). This "map" is not intended to show the actual sequence of reactions during catalysis, and omits a description of the catalytically important 3-electron form (Fig. 10, Reactions F and G). Reaction with NADPH produces the NADPH-AR-ADX complex, in which the strong binding of NADPH to the reduced flavoprotein produces a more favorable free energy change than that for reduction of flavoprotein in the absence of NADPH binding (7). The AR-ADX species is then formed in an unfavorable equilibrium with AR-ADX.

The reduced adrenodoxin thus formed, may then react with cytochrome c (Fig. 10, Reaction D). Fig. 11 also demonstrates that reduction of the first cytochrome c provides sufficient energy to "pull" Reaction C, Fig. 10 by removing AR-ADX from the equilibrium. The indicated energy "hill" in Fig. 11 could be significantly lowered if NADPH binds tightly to this species. This uncertainty is signified by the "question mark in" Fig. 11.

Reactions C plus D in Fig. 10 proceed with an overall apparent first order rate of 4.6 s\(^{-1}\) (see Table III), near the rate expected for the rate limiting step for the catalytic cycle (turnover = 3.2 s\(^{-1}\)). As shown in Table III, Experiment 2, a doubling of the cytochrome c concentration does not affect the rate of the rapid phase. This is in good agreement with studies using catalytic adrenodoxin reductase plus adrenodoxin, in which the rate of cytochrome c reduction by NADPH was found to be independent of cytochrome c concentration (8), and suggests a significant catalytic complex with cytochrome c in this concentration range.

The product of Reactions A through D in Fig. 10 should be the 1-electron-containing form of the adrenodoxin reductase-adrenodoxin complex. This form, also produced by reaction of reduced adrenodoxin with adrenodoxin reductase, has been shown in these studies to bind NADPH 2500-fold less tightly than the 2-electron-reduced form, suggesting that formation of this species in Reactions A through D should allow dissociation of NADPH from AR-ADX, as written in Reaction E of Fig. 10.

The 1-electron-containing species is shown to be but slowly reactive with cytochrome c (k = 0.11 to 0.33 s\(^{-1}\)). This lack of reactivity is not due to unfavorable free energy of reaction, since essentially all the cytochrome c is reduced in Experiment 2, Table III. The calculated free energies of this reaction are summarized in the right hand side of Fig. 11, and demonstrate the favorable energies for this reaction. Thus, the diminished reactivity of this species, compared to the 2-electron-reduced species, must represent a diminished accessibility of cytochrome c to the remaining electron. However, inclusion of a second NADPH allows rapid reduction of a second and third cytochrome c (Table III, Experiments 2 and 4), indicating the participation of Reactions F and G (Fig. 10) in the catalytic mechanism, as discussed above under "Results."

Thus, NADPH-cytochrome c reduction is catalyzed by the adrenodoxin reductase-adrenodoxin complex cycling between 1- and 3-electron-reduced forms. The fully oxidized form of the complex is observed only prior to reaction with the first NADPH, or following depletion of NADPH with a final slow oxidation of the 1-electron-containing form by remaining cyto-

\[\text{As previously stated under "Results," the precise value depends upon the extinction coefficient assumed for flavin semiquinone.}\]
The binding constant for the high affinity binding site NADP+ exists on the flavoprotein, as shown by the data of Figs. 7 and 8. The progress of the reaction occurs left to right. The vertical distance between any two solid bars is a measure of the free energy changes between the sets of components associated with those solid bars.

The low affinity site (Kd = 200 PM) is also on the flavoprotein, but not ferricyanide, reduction. Chu and Kimura have previously suggested a second pyridine nucleotide-binding site to account for this inhibition (8). This has been directly demonstrated in the present studies: at least two binding sites for NADP+ when NADPH is varied.6 However, catalytic studies demonstrate a "mixed" pattern of inhibition by NADP+, indicating not only a competitive component but also a noncompetitive component of NADP+ inhibition. The present studies show that the latter component is observed in cytochrome c, but not ferricyanide, reduction. Chu and Kimura have previously suggested a second pyridine nucleotide-binding site to account for this inhibition (8). This has been directly demonstrated in the present studies: at least two binding sites for NADP+ exist on the flavoprotein, as shown by the data of Figs. 7 and 8. The binding constant for the high affinity binding site (Kd = 13 PM) corresponds relatively closely to the Kd for NADP+ inhibition of adrenodoxin reductase-catalyzed NADPH-ferricyanide reductase activity (Kd = 24 PM). Since the latter adrenodoxin-independent activity demonstrates a competitive rather than a mixed pattern of inhibition by NADP+, the high affinity site must represent the site for reduction by NADPH of the FAD in adrenodoxin reductase. The low affinity site (Kd = 200 PM) is also on the flavoprotein, but appears to affect the flavoprotein-iron sulfur protein interaction (Fig. 7). Since only the adrenodoxin-dependent activity (cytochrome c reduction) demonstrates the noncompetitive component of inhibition by NADP+, we propose that the low affinity NADP+-binding site participates in the adrenodoxin reductase-adrenodoxin interaction.

Cytochrome P-450, the natural hemoprotein electron acceptor, requires the input of 2 electrons per heme in order to carry out steroid hydroxylation. Studies by Sligar et al. (25) have indicated that in electron transport to the bacterial cytochrome P-450 (31, 32). The interaction of two flavin groups in flavoproteins has been proposed by Hammerich (discussed in Ref. 32) to allow not only the splitting of an electron pair into single electrons, but also donation of each electron at constant oxidation-reduction potential. This function has been proposed for the FAD-FMN pair in sulfite reductase (32), and may function similarly in the microsomal cytochrome c (cytochrome P-450) reductase. Since adrenodoxin reductase contains only a single flavin, two sequential electron transfers from the reduced flavin group to ferricyanide must occur from electrons at two different oxidation potentials, i.e. those of the oxidized/semiquinone and semiquinone/reduced flavoprotein couples (approximately -360 mV and -230 mV, respectively). However, formation of the adrenodoxin reductase-adrenodoxin complex provides a means by which 2 electrons from NADPH may be delivered sequentially at a constant potential (i.e. that of the iron-sulfur group) to the acceptor cytochrome c or cytochrome P-450.

The rate equation for a slightly simplified but kinetically equivalent version of this mechanism has been derived by us using the method of King and Altman (24). Lineweaver-Burk plots constructed using this equation show a pattern of competitive inhibition by NADP+ when NADPH is varied.
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te and rubredoxin (33), ferredoxin:NADP+ oxidoreductase-ferredoxin (34), and have been suggested from catalytic studies of putidaredoxin and putidaredoxin reductase (35). In the case of ferredoxin:NADP+ oxidoreductase-ferredoxin, the complex has been suggested to be catalytically important (34). Although no studies in these systems have defined the state of association of the flavoprotein and nonheme iron protein during the catalytic cycle, one might conjecture that in these systems the complex also remains associated throughout a catalytic cycle, and may therefore function in a manner similar to the adrenodoxin reductase·adrenodoxin complex.

In summary, adrenodoxin reductase carries out adrenodoxin-dependent and adrenodoxin-independent reduction of l-electron acceptors with NADPH as electron donor. The catalytic mechanism depends upon the electron acceptor: adrenodoxin reductase cycles between oxidized and 2-electron-reduced forms for ferricyanide reduction, whereas the adrenodoxin reductase·adrenodoxin complex cycles between 1- and 3-electron-reduced forms for cytochrome c reduction.

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