Studies on Adrenal Steroid Hydroxylases. Oxidation–Reduction Properties of Adrenal Iron–Sulfur Protein (Adrenodoxin)†

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ABSTRACT: The adrenal iron–sulfur protein (adrenodoxin) was titrated to complete reduction with sodium dithionite, with reduced methylviologen, or with NADPH in the presence of adrenodoxin reductase. It is concluded from the results that adrenodoxin accepts one reducing equivalent. The same conclusion was also obtained by the oxidative titration of reduced adrenodoxin with 2,6-dichlorophenolindophenol, ferricyanide, or oxidized cytochrome *c*. The oxidation–reduction potential of adrenodoxin was determined by the dye–equilibrium reactions. The midpoint potential was found to be −0.27 V at pH 7.0. Previously reported values of the potential appear to be incorrect.

A ferredoxin-like iron–sulfur protein (adrenodoxin) has previously been isolated from adrenal cortex mitochondria (Suzuki and Kimura, 1965; Omura *et al.*, 1965). Physiologically this protein functions as an electron carrier for the steroid hydroxylation reactions in adrenal cortex, testis, and ovary mitochondria. It has been established that bovine adrenodoxin contains 2 g-atoms of iron and 2 mol of labile sulfur/mol of the protein, and has a molecular weight of 12,500 with a single polypeptide chain consisting of 114 amino acid residues.[1] The absorption spectrum of the oxidized protein shows distinct peaks at 455, 414, 320, and 276 nm with shoulders around 510 and 550 nm. Upon reduction, the absorbance is markedly bleached except for a maximum at 550 nm. From present data it is most likely that the structure of the iron center is binuclear tetrahedral.

In order to understand the electron transferring mechanism of steroid hydroxylation reactions, it is important to know the numbers of electrons transferred and the oxidation–reduction potential of this protein. Literature values for the oxidation–reduction potential and the number of electrons involved are controversial. Kimura and Suzuki (1967) obtained a midpoint potential of +164 mV with an *n* value of 2 by potentiometric titration with dithionite using a platinum electrode. However, only one-half of the total iron content in this protein could be accounted for by the intensity of the electron paramagnetic resonance (epr) signal of enzymatically reduced adrenodoxin when Cu–EDTA was used as a standard (Watari and Kimura, 1966). Later, Orme-Johnson and Beinert (1969) monitored the epr signal intensities of reduced adrenodoxin by titrating with diluted solid dithionite and obtained an *n* value of 1. Reinvestigation of the potential by Estabrook (1968) indicated a value of −367 mV by the spectrophotometric determination of a dye–adrenodoxin coupled reaction.

The purpose of this investigation was to investigate the discrepancy among the reported results.

Methods and Materials

NADP, NADPH, cytochrome *c* (type III), 2,4-dichlorophenolindophenol, FAD, and riboflavine were obtained from Sigma. Phenosafranine, Safranine T, Safranine O, methylviologen, and benzylviologen were from Aldrich and K & K. All safranines were recrystallized from the methanol solutions before use. In order to make sure that cytochrome *c* was fully oxidized, it was treated with ferricyanide, then passed through a G-25 column.

Adrenodoxin from bovine adrenals was prepared by the method described elsewhere (Kimura, 1968). The pure protein was kept either in the frozen state or as a lyophilized powder at −20°. The ratio of the absorbance at 414–276 nm was 0.86. The extinction coefficient of 11 mM^{-1} cm^{-1} was used for estimating the concentration of adrenodoxin.

Adrenodoxin reductase was isolated from bovine adrenal cortex mitochondria by the method of Omura *et al.* (1966) with some modifications. The ratio of the absorbance at 280–450 nm was 11.

The reduced dyes were prepared by the catalytic hydrogenation reaction in the presence of a platinized asbestos.

The anaerobic titration was performed in a glass apparatus which is similar to that described by Foust *et al.* (1969). A modified apparatus with two burets and two reservoirs was also constructed. The sodium dithionite solution was standardized with riboflavine under anaerobic conditions. The concentration of the riboflavine solution was determined spectrophotometrically by the use of the extinction coefficient of 12.5 mM^{-1} cm^{-1} at 455 nm.

In order to examine whether or not the dyes used bind to adrenodoxin, difference spectrophotometry was carried out by the use of tandem cuvets with two compartments in the light path. In the sample cuvet, the mixture of adrenodoxin and the dye was added to both compartments, and the reference cuvet contained adrenodoxin in one compartment and the dye in the other. We found that the dyes used in this study do not bind to adrenodoxin. Similar experiments with reduced adrenodoxin have been done, indicating no binding of the reduced dyes to the reduced protein.

The oxidation–reduction potential was calculated from eq 1, where *R* is the gas constant; *T*, the absolute temperature; *F*, the faraday constant; and *n*, the number of electrochemical equivalents. The midpoint potential of adrenodoxin

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TABLE I: Number of Electrons Transferred by Adrenodoxin Obtained from Oxidative and Reductive Titrations.

Method	Titration Reagents	n
Reductive titration of oxidized adrenodoxin	Sodium dithionite[a]	1.03
	NADPH[b]	1.04
	Reduced methylviologen[c]	1.16
Oxidative titration of adrenodoxin reduced by dithionite	2,6-Dichlorophenolindophenol[d]	0.96
	Ferricyanide[e]	1.00
	Oxidized cytochrome c[f]	1.02
Oxidative titration of adrenodoxin reduced by hydrogen gas	2,6-Dichlorophenolindophenol[d]	1.01
	Ferricyanide[e]	1.03
	Oxidized cytochrome c[f]	1.10

[a] Adrenodoxin, 5.1×10^{-5} M; sodium dithionite, 1.8×10^{-3} M. [b] Adrenodoxin, 4.7×10^{-5} M; NADPH, 1.3×10^{-3} M; and a catalytic amount of adrenodoxin reductase. [c] Adrenodoxin, 5.9×10^{-5} M; reduced methylviologen, 3.8×10^{-3} M. [d] Adrenodoxin, 5.0×10^{-5} M; 2,6-dichlorophenolindophenol, 1.10×10^{-2} M. [e] Adrenodoxin, 4.7×10^{-5} M; ferricyanide, 6.66×10^{-3} M. [f] Adrenodoxin, 4.2×10^{-5} M; cytochrome c, 7.85×10^{-3} M.

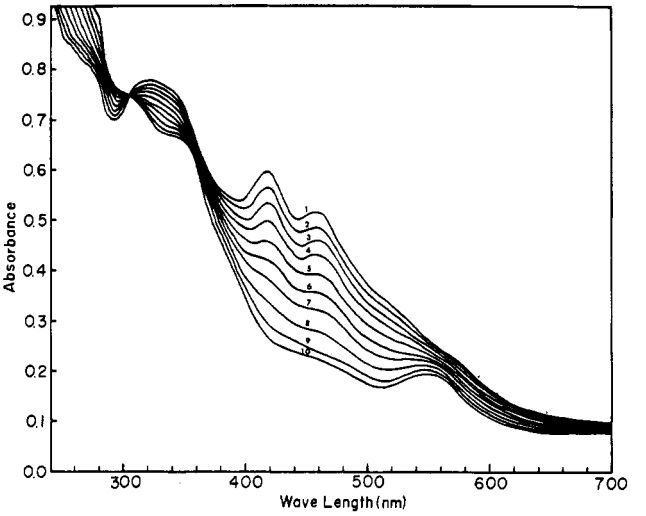

FIGURE 1: Titration of adrenal iron-sulfur protein (adrenodoxin) with sodium dithionite. A solution containing 5.23×10^{-5} M adrenodoxin, 5×10^{-9} M benzylviologen, and 0.05 M sodium phosphate buffer (pH 7.4), in a total of 2.80 ml, was titrated in an anaerobic cuvet with 5.2×10^{-3} M sodium dithionite solution. The spectra were not corrected for dilution. The amounts of dithionite added, in moles of dithionite/moles of adrenodoxin, were as follows. Curve 1, 0; curve 2, 0.055; curve 3, 0.099; curve 4, 0.180; curve 5, 0.204; curve 6, 0.272; curve 7, 0.324; curve 8, 0.391; curve 9, 0.459; curve 10, 0.503.

$$E_h = E_m7(\text{dye}) + \frac{RT}{n\text{F}} \ln \frac{[\text{oxidized dye}]}{[\text{reduced dye}]}$$

$$= E_m7(\text{adrenodoxin}) + \frac{RT}{n\text{F}} \ln \frac{[\text{oxidized adrenodoxin}]}{[\text{reduced adrenodoxin}]} \quad (1)$$

(E_m 7) was then obtained from a plot of E_h against logarithm of percentage reduction of adrenodoxin using eq 2.

$$E_m7(\text{adrenodoxin}) - E_m7(\text{dye}) = \frac{RT}{n\text{F}} \ln K_{eq} \quad (2)$$

The reduction of NADP was measured by a fluorometric method with the excitation wavelength of 360 nm and the emission wavelength of 460 nm. The extinction coefficients of the dyes used were separately measured by titrating the dyes with the standardized dithionite solution. The coefficients found are as follows: reduced methylviologen, 14 $\text{mM}^{-1}\ \text{cm}^{-1}$ at 602 nm; Safranines T and O, 48.0 $\text{mM}^{-1}\ \text{cm}^{-1}$ at 520 nm; and phenosafranine, 47.7 $\text{mM}^{-1}\ \text{cm}^{-1}$ at 520 nm.

Results

Reduction with Dithionite, NADPH and Adrenodoxin Reductase, and Reduced Methylviologen. In an anaerobic experiment with a catalytic amount of benzylviologen, the absorption peaks at 414 and 320 nm were gradually bleached as the dithionite solution was added (Figure 1). An isosbestic point at 306 nm was evident. The absorbance of 414 nm decreased to 40% of the oxidized form when the protein was fully reduced, and a peak appeared at 550 nm.

Adrenodoxin is reduced by NADPH in the presence of adrenodoxin reductase. The spectral changes during the titration with NADPH were similar to those shown in Figure 1. However, the absorbance at 414 nm showed 50% of the initial value, when 0.5 mol of NADPH/mol of adrenodoxin was added to the reaction mixture.

Adrenodoxin was then titrated anaerobically with reduced methylviologen. From these titration curves, the number of electrons transferred was calculated. The summary is shown in Table I.

Oxidation of Reduced Adrenodoxin with 2,6-Dichlorophenolindophenol, Ferricyanide and Cytochrome c. Reduced adrenodoxin was titrated anaerobically with 2,6-dichlorophenolindophenol, ferricyanide, or oxidized cytochrome c. The oxidation of reduced adrenodoxin was followed by measuring the increase in the absorbance at 414 nm. With the three oxidants, it was shown that the reduced protein functions as a one-electron donor. In Table I, the number of electrons transferred by adrenodoxin is summarized.

Titration of Adrenodoxin at Different pH's and Ionic Strengths. Adrenodoxin was titrated with dithionite at pH 5.5–10 and in 0.01–1.0 M KCl. The number of electrons transferred per mole of adrenodoxin was affected neither by pH nor by ionic strength.

Titration of Adrenodoxin in Urea and KCl. Since it has been shown that KCl stabilizes the iron–sulfur chromophore of adrenodoxin (Kimura and Nakamura, 1971), we have carried out a titration of adrenodoxin in a solution of 4 M urea, 1.0 M KCl, and 10 mM sodium phosphate buffer (pH 7.4), where the polypeptide chain is largely unfolded. The results were the same as in the case without urea and KCl. The number of electrons transferred by adrenodoxin is, therefore, not affected by these reagents. It was noticed that reduced adrenodoxin in KCl has a much slower rate of reoxidation by air, compared with that of reduced adrenodoxin without KCl.

Oxidation–Reduction Potential with Indicators. A typical example of the potential measurements is shown in Figure 2. With Safranine T, the midpoint potential of adrenodoxin was found to be −273 mV. The results of this type of experiment are summarized in Table II. By using the midpoint potential

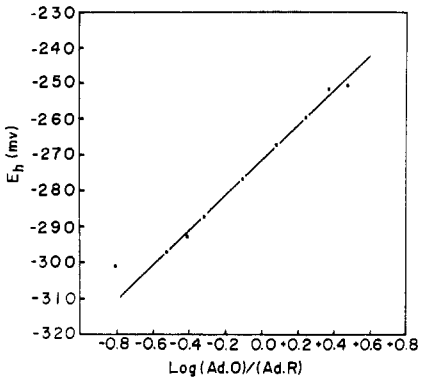

FIGURE 2: Oxidation-reduction potentials from the titration of adrenodoxin by Safranine T. Experimental conditions were 3.4×10^{-5} M adrenodoxin, 3.1×10^{-5} M Safranine T in 2.80 ml of 0.05 M sodium phosphate, pH 7.0 at 25°. The mixture was titrated by sodium dithionite (3.5 mM). E_h was calculated from eq 1 in the Methods and Materials section.

of −270 mV for adrenodoxin at pH 7.0, and that for NADPH of −320 mV, the equilibrium constant, K_{eq}, was calculated to be 39.2. In this table, it is noted that the midpoint potential calculated from the titration of adrenodoxin with anthroquinone-2-sulfonate was −249 mV, which is substantially lower than the values obtained with the other indicators. This low value is probably due to the fact that the rate of the reaction was very low and the calculation of the per cent of reduction had a large deviation.

Discussion

In the presence of a mediator such as benzylviologen, adrenodoxin was readily reduced by dithionite. In the absence, the rate of the reduction was very slow. At pH 7.0 and 25°, it took about 3 hr to reach complete equilibrium. This is, perhaps, the reason for obtaining incorrect values of the E_m and the number of electrons transferred by the potentiometric titration method, when complete equilibria were not attained (Kimura and Suzuki, 1967).

Precautions have to be taken when the method of a dye-coupled reaction is used for the determination of the oxidation-reduction potential. Before choosing the indicator, we examined a number of dyes with different chemical natures and oxidation-reduction potentials with respect to their nonspecific binding to adrenodoxin. They were tested by difference spectroscopy and by the gel filtration technique. We found no binding of the dyes used in this study of adrenodoxin. A difference of more than 50 mV in oxidation-reduction potential between the dye and adrenodoxin was found to be inappropriate. In our hands, dyes such as Brilliant Alizarine Blue (−173 mV) and viologens (about −400 mV) failed to give a reliable measurement. We believe, therefore, that the value of −367 mV reported by Estabrook's group is not correct.

2Fe-2S proteins, to which adrenodoxin belongs, closely resemble each other in terms of their optical absorption, circular dichroism, electron paramagnetic resonance, and Mössbauer spectroscopy. Although it is not clear why only one electron can be added to the 2Fe-2S center, this class of proteins functions as one-electron carriers. Our attempts to produce a two-electron reduction of adrenodoxin were unsuccessful under a variety of conditions such as different pH's, ionic strengths, and the presence of protein denaturants.

TABLE II: Oxidation-Reduction Potential of Adrenodoxin.[a]

Indicators	E_m7 of Indicator (mV)	No. of Expt	E_m7 of Adrenodoxin[b] (mV)
Anthroquinone-2-sulfonate	−225	2	−249 ± 10
FAD	−225	3	−268 ± 1.5
Safranine O	−253	2	−271 ± 1.2
Phenosafranine	−255	3	−267 ± 1.7
Safranine T	−289	3	−272 ± 1.3
NADPH[c]	−320	3	−272 ± 2.0
NADP[d]	−320	3	−273 ± 1.8

[a] A mixture containing approximately the same concentration of adrenodoxin and dye was made anaerobic in the titration apparatus. Small increments of sodium dithionite were added from the buret, and per cent reduction of adrenodoxin and dye was determined. Oxidized and reduced safranines showed an isosbestic point at 404 nm. Per cent reduction of adrenodoxin was calculated, therefore, from the decrease in the absorbance at this wavelength. Per cent reduction of the dyes was calculated from the decrease in the absorbance at 523 nm, after correcting for the absorbance of adrenodoxin at this wavelength. In the case of anthroquinone-2-sulfonate, per cent reduction of the dye was measured at 306 nm and that of the reduction of adrenodoxin was determined at 354 nm, where the quinone had an isosbestic point and also at 600 nm, where the quinone had no absorption. [b] [mean] ± [standard deviation]. [c] In the presence of adrenodoxin reductase. [d] In the presence of adrenodoxin reductase and dithionite.

The resemblance between adrenodoxin and other 2Fe-2S proteins ends when considering their oxidation-reduction potentials. The potentials of 2Fe-2S proteins range from −430 mV for spinach ferredoxin to −235 mV for putidaredoxin (Tagawa and Arnon, 1968; Wilson, 1969). The difference might reflect the microheterogeneity around the iron-sulfur active center.

Finally, it is of interest to compare the oxidation-reduction potential of adrenodoxin (−270 mV) with that of putidaredoxin (−235 mV). Both proteins have a very similar iron-sulfur chromophore as judged by various physical properties. Recent studies on the amino acid sequences of the two proteins (Tanaka *et al.*, 1970; Tsai *et al.*, 1971) further indicated a substantial similarity. However, it was shown by us (Kimura and Ohno, 1968) that putidaredoxin is not capable of substituting for adrenodoxin in steroid hydroxylation reactions. Lipscomb *et al.* (1972) recently demonstrated that adrenodoxin is not able to replace putidaredoxin in the camphor hydroxylation reaction of *Pseudomonas putida*. The nonexchangeability for the enzymatic activities may be due to a specificity of interaction of the iron-sulfur protein with the reductase and cytochrome P-450, rather than to the small difference in the potentials of adrenodoxin and putidaredoxin.

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On the Reaction of Purified Bovine Thrombin with *N*-Acetylimidazole†

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ABSTRACT: The reaction of thrombin with the mild acetylating agent, *N*-acetylimidazole, has been studied to further elucidate the relationship between structure and function in this highly specific proteolytic enzyme. Contrary to previous findings from other laboratories, the reaction of *N*-acetylimidazole with purified bovine thrombin resulted in an enzyme preparation with markedly decreased fibrinogen-clotting activity but apparently unchanged activity in the hydrolysis of tosyl-L-arginine methyl ester. The enzyme was reactivated upon treatment with either neutral hydroxylamine or exposure to mild alkaline conditions. The partial inactivation of the fibrinogen-clotting activity was associated with the acetylation of four to five tyrosyl residues as determined by spectrophotometric techniques or by estimation of hydroxamate formation after treatment with alkaline hydroxylamine. The enzyme was partially protected from inactivation by the presence of the competitive inhibitor, benzamidine hydrochloride, or by ester substrates. The inactivation by *N*-acetylimidazole was also prevented by tosyl-L-arginine and thus differed from the previously observed inactivation by phenylmethanesulfonyl fluoride. This information is used to support the hypothesis that inactivation of thrombin by *N*-acetylimidazole occurs at the macromolecular substrate binding site. Inactivation to a lesser degree has been observed with "aged" preparations of thrombin. The reaction of "aged" thrombin with *N*-acetylimidazole was associated with the acetylation of six to seven tyrosyl residues, suggesting a change in conformation since the process of "aging" is not apparently associated with autolysis.

The nature of the factors governing the highly specific catalytic action of thrombin (EC 3.4.4.13) are only poorly understood at this time. The use of diisopropyl phosphorofluoridate (Gladner and Laki, 1956; Miller and van Vunakis, 1956) and phenylmethanesulfonyl fluoride (Lundblad, 1971) has indicated the presence of a seryl residue at the catalytic site of thrombin. Glover and Shaw (1971) utilized an active-site-directed inhibitor, 1-chloro-3-tosylamido-7-amino-2-heptanone, to demonstrate the catalytic essentiality of a single histidyl residue. Since the above reagents diminish equally both the esterase and proteinase (fibrinogen-clotting) activity of highly purified preparations of thrombin, it is assumed that the affected residues participate in the actual catalytic reaction rather than in substrate binding or maintenance of proper conformation.

There are, however, conditions where differential stabilities of thrombin esterase and proteinase activity are observed. Sokolovsky and Riordan (1969) reacted crude preparations of bovine thrombin with tetranitromethane and observed that clotting activity was lost more rapidly and to a greater extent than esterase activity. This observation was extended by Lundblad and Harrison (1971) who utilized a highly purified preparation of bovine thrombin. These investigators reported a loss in fibrinogen-clotting activity upon treatment of thrombin with tetranitromethane but the esterase activity was apparently unchanged. The effect of acetylation of throm-

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