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THE EFFECT OF pH ON THE FORMAL REDUCTION POTENTIAL OF ADRENODOXIN IN THE PRESENCE AND ABSENCE OF ADRENODOXIN REDUCTASE: THE IMPLICATION IN THE ELECTRON TRANSFER MECHANISM

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We have investigated the formal reduction potentials (E°') of adrenodoxin with and without adrenodoxin reductase in order to elucidate the mechanism of electron transfer from adrenodoxin reductase (a flavoprotein) to adrenodoxin (an iron-sulfur protein). It was found by our spectropotentiostatic method that adrenodoxin showed no variation of E°' at different pH's in the absence of adrenodoxin reductase. The average E°' was -252±2 mV in the pH range between 6.0 and 8.3. In the presence of adrenodoxin reductase, adrenodoxin exhibited, on the other hand, a pH dependence of E°' at pH higher than 7.2 with a slope of -59 mV per pH unit: Adrenodoxin molecule possesses one protonation site with a pKa of 7.2. Cyclic voltammograms of adrenodoxin additionally revealed that the reoxidation reaction of reduced adrenodoxin is very slow in the absence of adrenodoxin reductase, but that it is readily reoxidized in the presence of adrenodoxin reductase.

The AR:ADX* system provides an excellent model in studying the mechanism of electron transfer from a flavin to an iron-sulfur center. It is long known that the two proteins form a 1:1 molar complex (1), which is catalytically important (2,3). There are some reports concerning the oxidation-reduction potentials of both AR and ADX (1,3,4). Furthermore, Lambeth and Kamin described that the reduction potential of ADX is pH dependent with a pKa of 6.8 (5). The reduction reaction was implicated by them as simultaneous electron and proton transfer from the flavin site to the iron-sulfur center. During our course of studies on the E°' measurements of various biological redox proteins by utilizing an electrochemical method, we found that E°' of ADX was independent of pH. In order to solve the apparent discrepancy between the two independent observations, we examined the possible reasons, and have found that in the presence of AR the oxidation-reduction properties of the ADX molecule change in two ways: (i) the reduction potential becomes more negative, for example, by -35 mV at pH 7.5, (ii) the E°' value shows pH dependence with a

ABBREVIATIONS: AR, adrenodoxin reductase; ADX, adrenodoxin (X stands for the conjugate base of one ionizing amino acid residue of which pKa is 7.2); BV, benzyl viologen; E°', formal reduction potential in mV vs NHE; SCE, saturated calomel electrode; $E_{applied}$, potential applied across working electrode and SCE; OTTLE, optically transparent thin-layer electrode; i_p, peak current.

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slope of -59 mV per pH unit in basic solution. These results are consistent with the fact that upon complex formation of AR and ADX both AR and ADX protein structures change significantly (6). In this paper, we emphasize the importance of the AR:ADX complex in the reversible electron transfer reaction of ADX and the role of environmental pH in regulating the reactivity of ADX. We describe here, for the first time, comparison of $E^{o'}$ of free ADX with that of the AR:ADX complex by the identical method of potential determination.

MATERIALS AND METHODS

BV was purchased from either K & K, or Sigma. It was crystallized twice from cold methanol by the addition of ether and vacuum dried overnight. ADX and AR were purified from adrenal cortex mitochondria (7). The ratio of absorbance at 414/276 nm for ADX and 450/272 nm for AR were 0.84 and 0.13, respectively. The absorption extinction coefficients of ε_{414} = 9.8 $mM^{-1}cm^{-1}$ and ε_{450} = 10.9 $mM^{-1}cm^{-1}$ were used to calculate the concentration of ADX and AR, respectively (7). Both oxidation-reduction enzymes were stored at -20° C, and a minimal amount was thawed as required.

All the experiments were performed with a three-electrode system using an Amel Model 551 Potentiostat/Galvanostat equipped with an Amel Model 566 Function Generator. Absorbance changes at different potentials were monitored with a Cary 219 spectrophotometer. A Houston 2200-5-6 X-Y recorder was used to acquire cyclic voltammograms when desired. The reduction potentials ($E^{o'}$) of ADX were obtained from the Nernst equation:

$$E_{applied} = E^{o'} + \frac{RT}{nF} \ln \frac{[O]}{[R]}$$

where [O] and [R] stand for the oxidized and reduced form, respectively.

OTTLE cell assembly was made according to the procedures described by Heineman *et al* (8) with some modification (9). The anaerobic conditions were achieved by exchanging air with argon gas and by adding glucose, glucose oxidase, and catalase. The oxygen concentration was found to be less than 6 μM (or 98% of removal of O_2) after the manipulations.

RESULTS

Cyclic Voltammetry: Figure 1, curve *a* shows that in the presence of BV as an electron mediator, ADX molecule exhibits only a measurable cathodic voltammetric response and almost no anodic wave. When AR is present in the system, a well-defined voltammetric response can be achieved (curve *b*). The experiments performed in more acidic conditions gave the same results. It was concluded from these observations that AR greatly improved the reversibility of the electron transfer reaction of ADX molecule. The results shown in Figure 2 draw the same conclusion by a different way. Line *a* and line *b* show a linearity between reduction peak current and square root of scan rate for ADX and AR:ADX complex, respectively, in the range of scan rates between 0.2 and 10 mV/sec. Both have similar large slopes. This suggests that homogeneous reduction of these enzymes by reduced BV must be faster than the heterogeneous reduction of BV at electrode surface. Line *c* shows the small

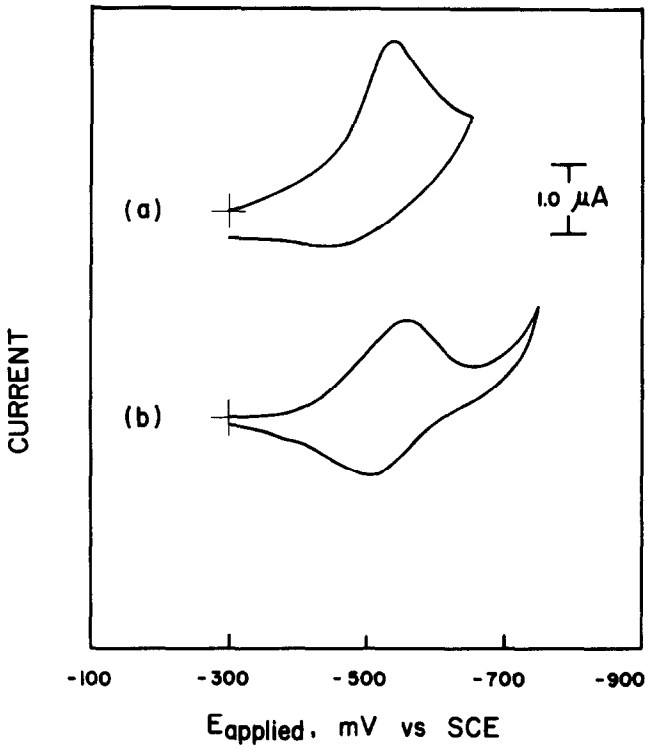

Figure 1. Cyclic voltammograms of ADX without (a) and with (b) the presence of AR at 25° C. The solution composition of (a) was 0.23 mM ADX, 0.01 mM BV, 0.10 M KCl, 0.10 M K-phosphate buffer, pH 7.4, and that of (b) was 0.23 mM ADX, 0.023 mM AR, 0.01 mM BV, 0.025 M KCl, 0.01 M K-phosphate buffer, pH 7.4. Both solutions contained 0.03 mg/100 μl glucose oxidase, 0.01 mg/100 μl catalase, and 0.1 M glucose as the oxygen scrubbing system. The scan rate was 0.5 mV/sec.

slope resulting from the reoxidation of reduced AR:ADX. The reoxidation current of ADX was too small to be measured. We conclude from these results that although reoxidation of ADX is slower than that of reduced BV radical, AR can accelerate ADX to a much faster rate which is comparable to that of reduced BV radical.

Spectropotentiostatic Experiments with Free ADX: In order to see whether the reduction of ADX involves the receipt of a proton as well as an electron, we have examined the pH effect of the reduction potential of ADX. Figure 3, curve a shows the results of free ADX at 25° C in the pH range 6.0 to 8.3 in the absence of AR. The E°' values obtained were invariant within our experimental errors, indicating that any protonation does not directly participate in the reduction reaction. The average E°' in this pH range was found to be -252±2 mV.

Spectropotentiostatic Experiments with an AR:ADX Complex: Most of the previous measurements of reduction potential of ADX were achieved by means of enzymatic reduction with NADPH in the presence of AR. Thus, in order to recon-

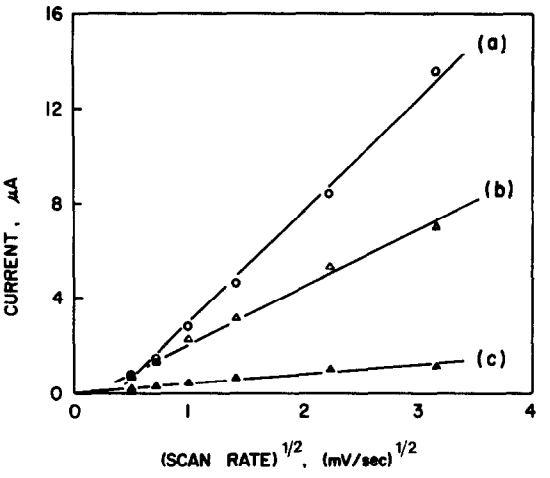

Figure 2. Scan rate dependence of cyclic voltammetric peak current: (a), reductive i_p of ADX; (b), reductive i_p of AR:ADX; (c), reoxidative i_p of reduced AR:ADX. Experimental conditions were the same as in Figure 1, except for the pH of ADX which was 6.8 and the scan rate varied from 0.2 mV/sec to 10 mV/sec.

cile the apparent discrepancy on the influence of pH, it is important to examine the effect of AR on ADX. When AR was present in a stoichiometric amount, E°' of ADX was constant at low pH whereas it became more negative at high pH

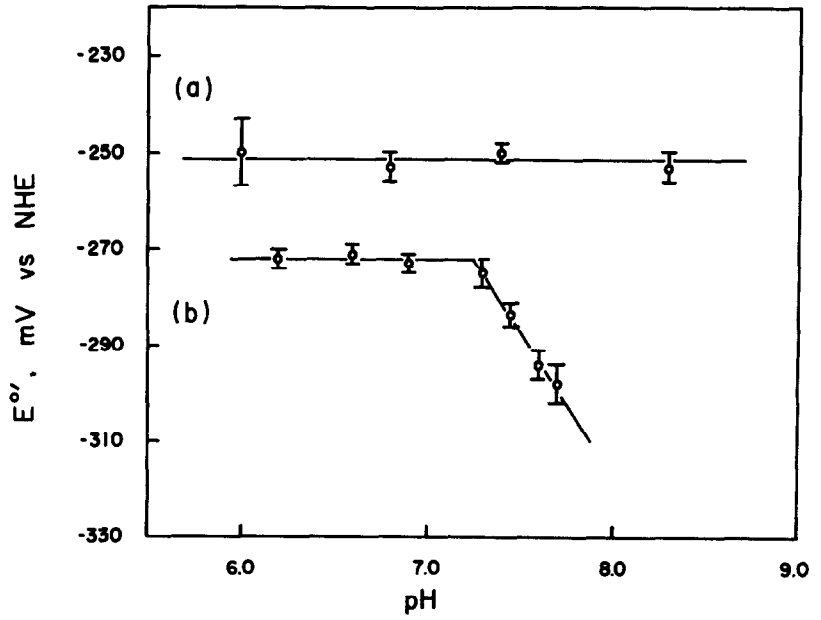

Figure 3. Variation of observed E°' of ADX in pH at 25° C. (a) 0.23 mM ADX in a solution containing 0.01 mM BV, 0.10 M KCl, 0.10 M K-phosphate buffer, (b) 0.23 mM ADX and 0.023 mM AR in a solution containing 0.01 mM BV, 0.025 mM KCl, 0.010 M K-phosphate buffer. The pH range of curve (b) was limited by the pH buffer capacity of 10 mM K-phosphate buffer and the stability of the AR:ADX complex.

with a slope of -59 mV per pH unit. The break point was at pH 7.2 (Figure 3, curve b). Lambeth and Kamin who used NADPH as a reductant obtained a break point at 6.8 and a slope with approximately -60 mV per pH unit (5). Our results show that at a physiological pH of 7.2 the ADX molecule in a 1:1 molar AR:ADX complex takes up one proton while it undergoes one-electron reduction. Figure 3, curve b shows that in the low pH region $E^{\circ\prime}$ of ADX was affected by -20 mV upon binding of AR, and it was affected by -35 mV at pH 7.5. A value of -40 mV at pH 7.5 was reported by Lambeth et al (3).

DISCUSSION

In normal catalytic functions of the adrenocortical steroid hydroxylase system, electron equivalents are transferred from NADPH to cytochrome P-450 via AR and ADX. In this study, the oxidation-reduction properties of ADX were examined with and without AR. Figure 1, curve a shows that ADX alone exhibits a very poor reoxidation step, while in curve b a greatly improved reoxidation process with the aid of AR. The poor reversibility of free ADX may be explained by differences in the pathway of electron transfer with respect to the reaction direction and by those of protein structures between oxidized and reduced adrenodoxin. When AR is present, a great improvement of the reversibility suggests the removal of these kinetical differences between reoxidative and reductive electron transfer reactions. Although the electron transfer pathway is heterogeneous from electrode to BV, followed by a homogeneous process to ADX or AR:ADX, Figure 2 indicates a heterogeneous electron transfer reaction. This fact suggests that reduction of ADX and AR:ADX by the reduced BV radical is much faster than that of BV by the electrode. It also implies that in the presence of AR the slow reoxidation rate of ADX becomes prompt and comparable to the reoxidation rate of reduced BV radical.

It was also found that thermodynamic properties of ADX were affected by AR. In the absence of AR, ADX has a single $E^{\circ\prime}$ value of -252±2 mV in the pH range between 6.0 and 8.3. It is concluded that any protonation, if any, does not participate in the reduction reaction of free ADX. This implies alternatively that both protonated (ADX-H) and unprotonated ADX have the same reduction potential:

$$ADX_{ox}\text{--}H \xrightarrow{e^-} ADX_{red}\text{--}H \qquad E^{\circ\prime} = -252 \text{ mV} \ldots\ (1)$$

$$ADX_{ox} \xrightarrow{e^-} ADX_{red} \qquad E^{\circ\prime} = -252 \text{ mV} \ldots\ (2)$$

In the presence of AR, the reduction potential of ADX ($E^{\circ\prime}_{obs}$) is pH dependent at pH higher than 7.2. The slope of -59 mV/pH shows that the ADX molecule takes up one proton while it undergoes one-electron reduction:

$$ADX_{ox} \xrightarrow{e^-,\ H^+} ADX_{red}\text{--}H \qquad E^{\circ\prime} \text{ is variable with pH} > 7.2. \quad (3)$$

On the other hand, the reduction potential of ADX ($E^{\circ\prime}*$) is pH-independent at pH below 7.2 even in the presence of AR. Under these conditions, the protonation reaction is not coupled with the electron transfer reaction:

$$ADX_{ox}\text{--}H \xrightarrow{e^-} ADX_{red}\text{--}H \qquad E^{\circ\prime}* = -272 \text{ mV at pH} < 7.2 \quad \ldots\ (4)$$

In the Equations 3 and 4, $E^{\circ\prime}*$ and $E^{\circ\prime}_{obs}$ are defined as follows:

$$E^{\circ\prime}_{obs} = E^{\circ\prime}* - (59 \text{ mV})\log(10^{pH-pKa}) \qquad \ldots\ (5)$$

Since adrenocortical mitochondria contain adrenodoxin reductase and adrenodoxin in approximately 1:10 molar ratio (7,10), our experimental system is similar to the AR and ADX content in mitochondria. In addition, it is known that the complex between the two proteins is catalytically important (2,3). The value of pKa 7.2 may have some physiological significance. Although the electron transfer mechanism from flavin to iron-sulfur center have been studied to some extent (5,11), we therefore conclude that both pH and AR play important roles in the modulation of ADX reactivity under physiological conditions. Recently, Lambeth and Pember observed that when ADX bound cytochrome $P\text{-}450_{scc}$ the reduction potential of ADX shifted from -273 to -291 mV (12) in a similar manner as the binding of ADX to AR. It is important to say that the protonation reaction is coupled with the electron transfer reaction only in the case of the Equation 3. Otherwise, the protonation is not coupled. At present, we do not have any direct evidence on the implication of the simultaneous proton and electron transfer mechanism from AR to ADX. It would be possible under the conditions of the Equation 3 only. It may be worth mentioning that based on electrochemical studies bacterial putidaredoxin displays two proton-linked processes in the oxidized form and one in the reduced form in the absence of its reductase (13). In spite of the fact that the similarity between ADX and putidaredoxin is striking, their properties of the proton-involving oxidation-reduction reaction are remarkably different.

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