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# Evidence for the Cluster Model of Mitochondrial Steroid Hydroxylase System Derived from Dissociation Constants of the Complex between Adrenodoxin Reductase and Adrenodoxin

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**Using biotinylated adrenodoxin and avidin–Sepharose 4B, dissociation constants for the complex between adrenodoxin reductase and adrenodoxin in the oxidized and reduced states were determined as 50 ± 11 and 296 ± 44 nM, respectively. Concentrations of adrenodoxin reductase/adrenodoxin in the matrix fraction from bovine adrenal cortex, liver, and kidney mitochondria were determined to be 20.2 ± 10.6 μM/120 ± 23 μM, 0.17 ± 0.06 μM/1.79 ± 0.24 μM, and 0.40 ± 0.23 μM/1.33 ± 0.26 μM, respectively. The calculation of the percentage of adrenodoxin reductase in the complex form in the reduced state showed that it is higher than 99% in adrenal cortex mitochondria, providing clear evidence for the cluster model for the mitochondrial steroid hydroxylase system.** © 2000 Academic Press

Steroid hydroxylase system of mitochondria in adrenal cortex, ovary, testis, and placenta consists of NADPH-adrenodoxin reductase (AR), adrenodoxin (AD), and cytochromes P450 (P450) (1). In this system, AD functions as an electron carrier between AR and P450 to transfer electrons for the steroid hydroxylation reactions (2, 3). To explain the mechanism for these electron transfer reactions, two models, the cluster and the shuttle mechanism, have been proposed (4–13). As the cluster model, the ternary complex model (7) and the quaternary complex model (12, 13) have been proposed. Lambeth *et al.* (4–6) proposed the shuttle mechanism in which AD functions as a mobile electron carrier between AR and P450. Their proposal is based on the observation that the dissociation constant ($K_d$) for AR–AD complex is increased 22 times by the reduction (6). However, the dissociation of the complex depends not only on the $K_d$ values but also on the concentrations of AR and AD. In this study, we determined the $K_d$ values for AR–AD complex in the oxidized and reduced states. The concentrations of AR and AD in the matrix fraction of mitochondria prepared from bovine adrenal cortex, liver, and kidney were also determined to deduce the percentage of AR in the complex form *in vivo.* From the theoretical calculation of the percentage of AR in the complex form, we present clear evidence for the cluster model of the reaction mechanism.

Abbreviations used: AD, adrenodoxin; AD-biotin(Lys), AD biotinylated with 6 lysyl residues; $[AD]_{total}$, concentration of total AD; AR, adrenodoxin reductase; $[AR]_b$, concentration of bound AR; $[AR]_f$, concentration of free AR; $[AR]_{total}$, concentration of total AR; $K_d$, dissociation constant; P450, cytochrome P450; P450scc, cytochrome P450 catalyzing cholesterol side chain cleavage reaction.

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## MATERIALS AND METHODS

*Purification of AR and AD.* Purification of AR from bovine adrenal cortex was carried out by the method of Ohashi and Omura (14) with some modification (11). Bovine AD was expressed in *Escherichia coli,* and purified as described (15).

*Biotinylation of AD.* To purified AD (250 nmol, $A_{414}/A_{276}$ = 0.910) in 20 ml of 50 mM potassium phosphate buffer (pH 8.0), 15 μmol of D-biotin-*N*-hydroxysuccinimide ester (Boehringer Mannheim Biochemica), 10 times molar excess over lysyl residues of AD, in 0.1 ml of dimethyl sulfoxide solution was added, and the mixture was stirred gently for 3 h at 25°C. The mixture was then applied onto a small DE-52 column (bed volume, 1 ml) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.4). The column was washed with the same buffer containing 0.1 M KCl, and eluted with the buffer containing 0.5 M KCl. The eluted biotinylated AD fraction was dialyzed overnight against 10 mM potassium phosphate buffer (pH 7.4) extensively at 4°C, and used as AD-biotin(Lys).

*Preparation of avidin–Sepharose 4B.* Three grams of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) were suspended in 50 ml of 1 mM HCl, filtered, and washed with 10 mM potassium phosphate buffer (pH 8.3). The washed resin was mixed with 20 ml of the same buffer containing 24 mg avidin (Wako Pure Chemical Industries, Japan). In some experiments, 6–18 mg of avi-

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din was used for the coupling. The reaction mixture was stirred gently for 3 h at 25°C. After filtration, the resin was further mixed with 20 ml of 1 M ethanolamine (pH 8.0), and stirred for 2 h at 25°C to block all of the unreacted sites of the resin. After washing the resin with 10 mM potassium phosphate buffer (pH 7.4), it was suspended in 30 ml of the same buffer.

*Determination of $K_d$ for AR–AD complex in the oxidized state.* The reaction tubes ($v$ = 1.5 ml) contained 0.8 $\mu$M AD-biotin(Lys) and various concentrations of AR (0.1–1.0 $\mu$M) in 0.5 ml of 10 mM potassium phosphate buffer (pH 7.4). As the control, the tubes without biotinylated AD were used for the calibration of AR. To all the tubes, 0.2 ml of the standard avidin-Sepharose 4B [24 times molar excess of avidin subunits over AD-biotin(Lys) molecule] was added, and the tubes were mixed thoroughly by more than 20 times of inversion. In some experiments, 0.2 ml of avidin–Sepharose 4B containing 6–16 times molar excess of avidin was used to check the concentration dependency of the determination. The tube was centrifuged at 1000 rpm for 1 min at 4°C. Under these conditions, neither free AD-biotin(Lys) nor AR–AD-biotin(Lys) complex was detected in the supernatant. AR concentration in the supernatant was determined by NADPH–cytochrome *c* reductase assay as follows. The cuvette contained appropriate amounts of the supernatant and 0.425 $\mu$M AD in 2 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 20 $\mu$M cytochrome *c* and 0.1 mM NADPH, and the reaction was started by the addition of NADPH, and the changes in absorbance at 550 nm were followed at 25°C.

*Determination of $K_d$ for AR–AD complex in the reduced state.* The reaction tubes ($v$ = 1.5 ml) contained 0.8 $\mu$M AD-biotin(Lys) and various concentrations of AR (0.1–1.0 $\mu$M) in 0.5 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 10 mM glucose, 20 $\mu$g/ml each of glucose oxidase and catalase (Sigma), and 0.1 mM NADPH. Before and after the addition of AR and biotinylated AD, the tube was sealed with a serum cap and the solution was thoroughly evacuated and flushed with nitrogen gas. To the tube, 0.2 ml of avidin–Sepharose 4B previously evacuated and flushed with nitrogen gas several times was injected by a syringe. After repeated inversions, the tube was centrifuged at 1000 rpm for 1 min at 4°C. AR concentration in the supernatant was determined as described above except that the reaction was started by the addition of cytochrome *c*.

*Determination of concentrations of AR and AD in the matrix fraction of mitochondria.* Bovine adrenal, liver, and kidney were obtained from a local slaughterhouse. Ten grams of adrenal cortex, liver, and kidney was homogenized with 9 volumes of 0.25 M sucrose containing 10 mM Tris–HCl buffer (pH 7.4) and 0.1 mM EDTA. Mitochondrial fraction was prepared by the method of Ohashi and Omura (14). The fluffy layer was discarded, and the weight of the mitochondrial pellet was measured. The pellet was resuspended with 10 ml of 10 mM potassium phosphate buffer (pH 7.4), and sonicated for 20 s three times with an interval of 20 s in an ice bath with a TOMY UD-201 ultrasonic disrupter. After centrifugation at 100,000*g* for 60 min, the supernatant was used as the matrix. The weight of the pellet was measured again, and the difference in the weight was assumed as the weight of matrix. The volume of matrix was calculated from the density of 1.3 for the soluble protein fraction (16). Concentrations of AR and AD were determined by the method of Ohashi and Omura (14).

*Other analytical methods.* Concentrations of purified AR and AD were determined from the extinction coefficients of 10.9 $mM^{-1}$ $cm^{-1}$ at 450 nm for AR (17) and 9.8 $mM^{-1}$ $cm^{-1}$ at 414 nm for AD (14), respectively. Reduced cytochrome *c* was calculated from 19.0 $mM^{-1}$ $cm^{-1}$ at 550 nm (5). Bound biotin in AD was determined by the method of Green (18). Protein was determined by the method of Lowry *et al.* (19) using bovine serum albumin as a standard. Scatchard plot analysis was carried out as described (20).

## RESULTS

*The principle of determination of $K_d$ and examination for the suitable conditions.* The vitamin biotin has been used for many methods to detect various biologically important substances using high affinity of biotin for avidin (21). To establish a new method for determination of $K_d$ of the complex between AR and AD, we have applied the biotin-avidin system. The principle of this method is based on biotinylation of AD, complex formation of the biotinylated AD with AR, and separation of free AR from the AR–AD complex with avidin–Sepharose 4B. Bovine AD molecule has 6 lysyl residues in 128 amino acids (22). Various chemical modifications of lysyl residues of AD have shown that such modifications affect neither NADPH–cytochrome *c* reduction nor cholesterol side chain cleavage reaction catalyzed by AR–AD complex (23). We prepared AD-biotin(Lys) using D-biotin–*N*-hydroxysuccinimide ester to modify lysyl residues of AD. After purification of AD-biotin(Lys), the ratio of $A_{414}$ to $A_{276}$ of AD-biotin(Lys) remained 0.86–0.88, indicating that the conformation of AD did not change significantly. More than 98% of lysyl residues of AD-biotin(Lys) were found to be biotinylated. The $K_m/V_{max}$ values for AD-(wild) and AD-biotin(Lys) in NADPH–cytochrome *c* reduction were 184 nM/904 $min^{-1}$ and 310 nM/1227 $min^{-1}$, respectively. Avidin has 4 subunits and the molecular mass is 67 kDa. NADPH–cytochrome *c* reduction catalyzed by AR and AD-biotin(Lys) was inhibited by 15% in the presence of 400 times molar excess of avidin subunits over AD-biotin(Lys) (data not shown). However, avidin–Sepharose 4B for full sedimentation of AD-biotin(Lys) in the reaction mixture was only 6–24 times molar excess of avidin subunits over AD-biotin(Lys). Since the inhibition of the reaction was less than 2% in this case, the effect of avidin on AR binding to AD-biotin(Lys) was negligible.

The most important problem to determine the $K_d$ value in the reduced state is the difficulty of the maintenance of anaerobic condition in the tube during the series of manipulation. We checked whether or not the reduced state of AR–AD complex is maintained by measuring the absorption spectra. The absorption maxima at 450 nm, 414 nm, and 330 nm, indicating the typical oxidized AR–AD complex (11), decreased after the reduction by NADPH. The spectrum of reduced AD as judged by the absorption maximum at 546 nm was maintained up to 150 min after the reduction, indicating that the anaerobic condition was kept during the assay of free AR in the supernatant by this method (data not shown). We found that the reduced state was not affected by re-aeration in the presence of glucose-glucose oxidase-catalase system.

*$K_d$s of AR–AD Complex in the oxidized and reduced states.* Figure 1 shows a typical determination of AR in the supernatant after precipitation of AD-biotin(Lys)

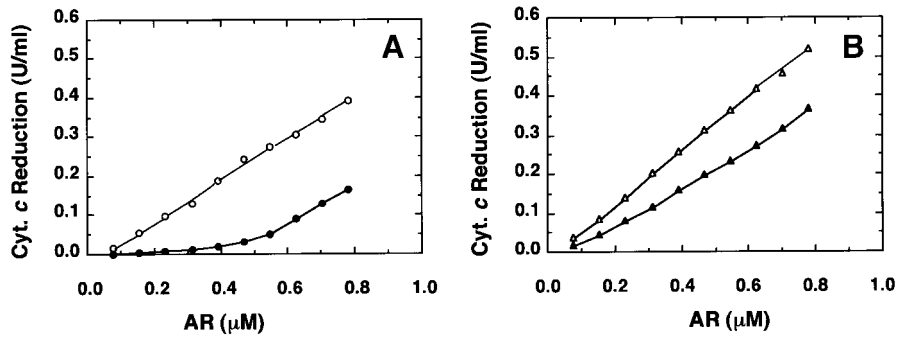


**FIG. 1.** Determination of AR in the supernatant after precipitation with avidin-Sepharose 4B in the presence or absence of AD-biotin(Lys) in the oxidized and reduced states. The reaction mixtures for the oxidized state (A) contained various concentrations of AR (0.1–1.0 $\mu$M) in the presence (●) or absence (○) of 0.8 $\mu$M AD-biotin(Lys) in 0.5 ml of 10 mM potassium phosphate buffer (pH 7.4). The reaction mixtures for the reduced state (B) contained various concentrations of AR (0.1–1.0 $\mu$M) in the presence (▲) or absence (△) of 0.8 $\mu$M AD-biotin(Lys) in 0.5 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 10 mM glucose and 20 $\mu$g/ml each of glucose oxidase and catalase. AR and AD-biotin(Lys) were reduced by NADPH, and the reduced state was maintained by glucose-glucose oxidase-catalase system as described under Materials and Methods. After the addition of avidin-Sepharose 4B, the tube was centrifuged, and AR in the supernatant was measured by the NADPH–cytochrome *c* reductase assay.

with avidin–Sepharose 4B in the oxidized and reduced states. In the reduced state (Fig. 1B), the amount of AR bound to AD-biotin(Lys) decreased to 60–70% of that of the oxidized state (Fig. 1A), suggesting that the reduction of AR–AD complex induced some dissociation of the complex. To analyze the changes in the complex formation, we performed Scatchard plot analysis, and $K_d$ values for the complex in the oxidized and reduced states were calculated to be 43 and 283 nM, respectively (Fig. 2). We used different concentrations of avidin-Sepharose 4B (1/4 to 2/3 of the standard assay) to check the concentration dependency of $K_d$ values. The data were not different from those of the standard assay. Table I shows $K_d$ values for AR–AD complex in the oxidized and reduced states determined from 3 separate experiments. The $K_d$ value in the reduced state was about 6 times larger than that of the oxidized state. This difference in the $K_d$ values between the oxidized and reduced states was significantly smaller than that (22 times) by Lambeth *et al.* (6) determined with an indirect method. After addition of avidin–Sepharose 4B, the corrected concentrations of total AD were 0.63–0.70 $\mu$M. [AR]b in the oxidized state was calculated to be 0.67 ± 0.04 $\mu$M, whereas that in the reduced state was 0.33 ± 0.01 $\mu$M. These results suggest that AR binds to AD-biotin(Lys) forming a 1:1 complex in the oxidized state and a 1:2 complex in the reduced state, respectively (Table I and Fig. 2).

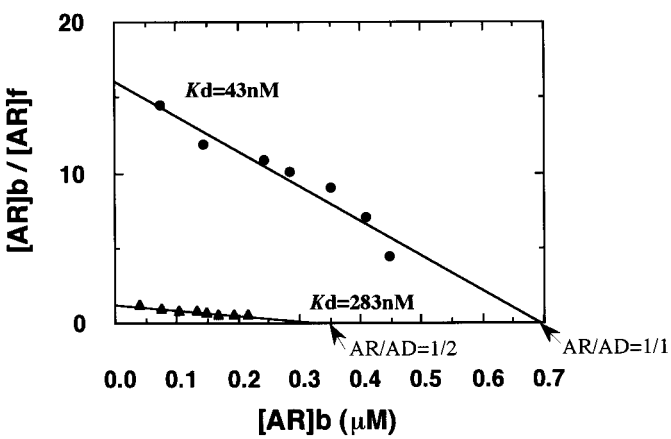


**FIG. 2.** Scatchard plot analysis of AR binding to AD-biotin(Lys) in the oxidized and reduced states. As shown in Fig. 1, the concentration of free AR ([AR]f) was determined from the amount of AR in the supernatant, while that of bound AR ([AR]b) was calculated by subtracting free AR from that of total AR under the oxidized and reduced conditions. The $K_d$ values for AR–AD complex in oxidized (●) and reduced (▲) states were calculated to be 43 and 283 nM, respectively. The concentrations of bound AR in oxidized and reduced states were calculated to be 0.70 and 0.35 $\mu$M, respectively.

*Determination of concentrations of AR and AD in mitochondrial matrix fraction and the theoretical concentration of AR in the complex form.* The $K_d$ value of AR–AD complex in the oxidized state is given by the

**TABLE I**

$K_d$ Values of AR–AD Complex in the Oxidized and Reduced States

| Redox states of AR and AD | $K_d$ values (this work) (nM) | $[AR]_b$ (this work) ($\mu$M) | $K_d$ values (Lambeth *et al.*] (nM) |
|---|---|---|---|
| AR(ox) AD(ox) | 50 ± 11[a] | 0.67 ± 0.04[a] | 25 |
| AR(red) AD(red) | 296 ± 44[a] | 0.33 ± 0.01[a] | 540 |

[a] Determined from three separate experiments expressed as the mean ± SD. AR(ox) and AD(ox) represent oxidized forms of AR and AD, respectively. AR(red) and AD(red) represent reduced forms of AR and AD, respectively. The volume of supernatant was measured because of increase in the volume after addition of avidin–Sepharose 4B, and the corrected concentrations of AD in the tube were 0.63–0.7 $\mu$M. The $K_d$ values reported by Lambeth *et al.* (6) are shown for comparison.

**TABLE II**

Determination of Concentrations and Specific Contents of AR and AD in the Matrix Fraction of Mitochondria Prepared from Bovine Adrenal Cortex, Liver, and Kidney

| | AR | | AD | |
|---|---|---|---|---|
| Organs | ($\mu$M) | ($\mu$g/mg protein) | ($\mu$M) | ($\mu$g/mg protein) |
| Adrenal cortex | 20.2 ± 10.6 | 30.3 ± 13.4 | 120 ± 23.0 | 49.7 ± 6.6 |
| Liver | 0.17 ± 0.06 | 0.27 ± 0.10 | 1.79 ± 0.24 | 0.80 ± 0.15 |
| Kidney | 0.40 ± 0.23 | 0.75 ± 0.39 | 1.33 ± 0.26 | 0.70 ± 0.11 |

*Note.* Each value represents the mean ± SD of four separate experiments.

following equations since AR binds to AD as a (1/1 = mol/mol) complex:

$$[AR][AD]/[AR–AD] = K_d \quad [1]$$

$$[AR]_{total} = [AR] + [AR–AD] \quad [2]$$

$$[AD]_{total} = [AD] + [AR–AD] \quad [3]$$

The $K_d$ value of AR–AD complex in the reduced state is given by the following equations since AR binds to AD as a (1/2 = mol/mol) complex:

$$[AR][(AD)_2]/[AR–(AD)_2] = K_d \quad [4]$$

Where $(AD)_2$ shows the AD dimer since n value of the ligand as AR in this experiment in the reduced state is 0.46–0.49 (Table I). We found that the complex between AR and the AD dimer was detected when AD was reduced by AR in the presence of NADPH, and the mixture of the reduced AR and AD was applied onto a Sephadex G-75 column in the presence of glucose-glucose oxidase-catalase system (data not shown). Therefore, Eq. [4] can be applicable for the calculation.

$$[AR]_{total} = [AR] + [AR–(AD)_2] \quad [5]$$

$$[AD]_{total} = [AD] + 2[(AD)_2] + 2[AR–(AD)_2] \quad [6]$$

If $[(AD)_2] \gg [AD]$, then Eq. [6] is

$$[AD]_{total} = 2[(AD)_2] + 2[AR–(AD)_2] \quad [7]$$

This assumption is reasonable because we found that the AD monomer was hardly detected by the gel filtration mentioned above. To calculate [AR–AD] or [AR–$(AD)_2$], $[AR]_{total}$ and $[AD]_{total}$ values are necessary. We therefore determined the concentrations of AR and AD in mitochondrial matrix fraction. As shown in Table II, the concentrations of AR and AD in the adrenal cortex mitochondria were very high. They were 20.2 ± 10.6 $\mu$M and 120 ± 23 $\mu$M for AR and AD, respectively. AR and AD are also known to exist in liver and kidney mitochondria, although their concentrations are supposed to be 1/50 to 1/100 of those of adrenal cortex (14). Concentrations of AR and AD in the matrix fraction of liver and kidney mitochondria were also determined to be 0.17 ± 0.06 and 1.79 ± 0.24 $\mu$M, and 0.40 ± 0.23 and 1.33 ± 0.26 $\mu$M, respectively. The specific contents of AR and AD in these organs are a little higher than those of Ohashi and Omura (14), but lower than those by Hamamoto *et al.* (24) who used Western blotting analysis.

**TABLE III**

Calculation of [AR–AD] and [AR $(AD)_2$] and % of AR in the Complex Form in the Matrix Fractions of Mitochondria Prepared from Bovine Adrenal Cortex, Liver, and Kidney

| Organs | Redox state | [AR–AD] ($\mu$M) | [AR–$(AD)_2$] ($\mu$M) | % of AR in the complex form |
|---|---|---|---|---|
| Adrenal cortex | Oxidized | 20.19 | — | 99.95 |
| | Reduced | — | 20.10 | 99.50 |
| Liver | Oxidized | 0.16 | — | 97.02 |
| | Reduced | — | 0.13 | 76.85 |
| Kidney | Oxidized | 0.38 | — | 95.00 |
| | Reduced | — | 0.24 | 59.14 |

*Note.* [AR–AD] was calculated from Eqs. [1], [2], and [3] using 50 nM as the $K_d$ value. [AR–$(AD)_2$] was calculated from Eqs. [4], [5], and [7] using 296 nM as the $K_d$ value. $[AR]_{total}$ and $[AD]_{total}$ were taken from Table II.

Table III shows [AR–AD] in the oxidized state and [AR–$(AD)_2$] in the reduced state calculated from Eqs. [1]–[7] with $[AR]_{total}$ and $[AD]_{total}$ values taken from Table II. In adrenal cortex mitochondria, more than 99% of AR is in the complex form even if the $K_d$ value increases from 50 nM to 296 nM after the reduction of AR. In the liver and kidney mitochondria, 77 and 59% of AR are calculated to be in the complex form in the reduced state, respectively. These results clearly indicate that the complex between AR and AD does not dissociate to a great extent during an oxidation-reduction cycle, providing clear evidence for the cluster model for mitochondrial steroid hydroxylase system.

## DISCUSSION

Two theories have been proposed for the reaction mechanism for mitochondrial steroid hydroxylase system (4–13), however, many unsolved problems still remain. For example, association and dissociation of the complex between AR and AD in the oxidized and reduced states should be quantitatively analyzed. Although physiological concentrations of AR and AD as well as the $K_d$ values determine the quantitation of complex, such data are not available. This study deals with these problems.

The $K_d$ values of AR–AD complex were 50 ± 11 and 296 ± 44 nM in the oxidized and reduced states, respectively. Tertiary structure analysis of crystallized truncated AD (4–108) (shown as amino acid numbers of AD molecule) indicates that five lysyl residues of AD (Lys-126 is absent) are located together at a remote site from the binding site of AR or P450scc (25). Therefore, it seems likely that the binding of avidin to AD-biotin(Lys) does not significantly interfere with the interaction between AR and AD. Thus the $K_d$ values of AR–AD complex in this study may be applicable to the native AR–AD complex.

The concentrations of AR and AD in mitochondrial matrix fraction from adrenal cortex were very high. The $K_d$ values in this study were determined with 10 mM potassium phosphate buffer, whose ionic strength is much lower than the physiological condition. The $K_d$ value for AR and AD complex increases by 10 times when the ionic strength of the medium expressed as NaCl concentration is shifted from 25 mM to 150 mM (4). We calculated that more than 93% of AR in adrenal cortex mitochondria is in the complex form even at the physiological ionic strength when we apply the 10 times larger $K_d$ value than that determined in this study. In either case, the high concentrations of AR and AD in the matrix fraction contribute to the complex formation even when the $K_d$ value increases by the ionic strength change. When the $K_d$ values determined by Lambeth *et al.* (6) are applied, % of AR in the complex form is higher than 99%. In the liver and kidney mitochondria, AR and AD concentrations were much lower than those of adrenal cortex (1/50 to 1/100 of those of adrenal cortex mitochondria). However, 77 and 59% of AR in the liver and kidney mitochondria, respectively, are calculated to be in the complex form in the reduced state. These values can be calculated to decrease to 17–22% under the physiological condition. However, it should be noted that the existence of the complex, even if the proportion is low, may have some significance for the regulation of the metabolism of bile acids (26) and vitamin D3 (27) catalyzed by AR and AD in these organs.

It is important to note here how much $K_d$ value is necessary for the drastic dissociation. If the drastic dissociation of AR–AD complex is defined as the decrease in % of AR in the complex form to 1%, the $K_d$ values in the reduced state for adrenal cortex, liver, and kidney mitochondria which are necessary for the drastic dissociation are calculated to be 6000, 85, and 60 $\mu$M, respectively. The differences in $K_d$ values in the oxidized and reduced states determined in this work and other work (6) are too small to induce the drastic dissociation of the complex upon reduction. The existence of high proportion of the complex even in the reduced state under physiological concentrations of AR and AD in adrenal cortex mitochondria indicates that the cluster model is more feasible for the reaction mechanism.

It is interesting that the Scatchard plot analysis of reduced AR–AD complex showed the existence of a 1:2 AR/AD complex. We detected the complex between AR and the AD dimer in the reduced state by gel filtration (Hara, T. *et al.*, unpublished observation). This finding may be related to the quaternary cluster mechanism, in which 1 mol each of AD binds to AR and P450 (12, 13). Although the clarification of structural and physiological significance of the AD dimer in relation to the quaternary cluster organization among AR, AD, and P450 must await further clarification, our findings in this work support the cluster model for mitochondrial steroid hydroxylation reactions.

## REFERENCES

1. Kimura, T. (1981) ACTH stimulation on cholesterol side chain cleavage activity of adrenocortical mitochondria. Transfer of the stimulus from plasma membrane to mitochondria. *Mol. Cell. Biochem*. **36,** 105–122.
2. Omura, T., Sato, R., Cooper, D. Y., Rosenthal, O., and Estabrook, R. W. (1965) Function of cytochrome P-450 of microsomes. *Fed. Proc.* **24,** 1181–1189.
3. Kimura, T., and Suzuki, K. (1967) Components of the electron transport system in adrenal steroid hydroxylase. Isolation and properties of non-heme iron protein (adrenodoxin). *J. Biol. Chem*. **242,** 485–491.
4. Lambeth, J. D., Seybert, D. W., and Kamin, H. (1979) Ionic effects on adrenal steroidogenic electron transport. The role of adrenodoxin as an electron shuttle. *J. Biol. Chem*. **254,** 7255–7264.
5. Lambeth, J. D., Seybert, D. W., and Kamin, H. (1980) Adrenodoxin reductase–adrenodoxin complex. Rapid formation and

breakdown of the complex and a slow conformational change in the flavoprotein. *J. Biol. Chem*. **255,** 4667–4672.

6. Lambeth, J. D., and Pember, O. S. (1983) Cytochrome P-450scc–adrenodoxin complex. Reduction properties of the substrate-associated cytochrome and relation of the reduction states of heme and iron-sulfur centers. *J. Biol. Chem*. **258,** 5596–5602.
7. Kido, T., and Kimura, T. (1979) The formation of binary and ternary complexes of cytochrome P-450scc with adrenodoxin and adrenodoxin reductase–adrenodoxin complex. The implication in ACTH function. *J. Biol. Chem*. **254,** 11086–11815.
8. Chashchin, V. L. Turko, I. V., Akhrem, A. A., and Usanov, S. A. (1985) Cross-linking studies of adrenocortical cytochrome P-450scc. Evidence for a covalent complex with adrenodoxin and localization of the adrenodoxin-binding domain. *Biochim. Biophys. Acta* **828,** 313–324.
9. Usanov, S. A., Turko, I. V., Chashchin, V. L., and Akhrem, A. A. (1985) Cross-linking studies of steroidogenic electron transfer: Covalent complex of adrenodoxin reductase with adrenodoxin. *Biochim. Biophys. Acta* **832,** 288–296.
10. Turko, I. V., Adamovich, T. B., Kirillova, N. M., Usanov, S. A., and Chashchin, V. L. (1989) Cross-linking studies of the cholesterol hydroxylation system from bovine adrenocortical mitochondria. *Biochim. Biophys. Acta* **996,** 37–42.
11. Hara, T., and Kumura, T. (1989) Purification and catalytic properties of a cross-linked complex between adrenodoxin reductase and adrenodoxin. *J. Biochem*. **105,** 594–600.
12. Hara, T., and Kumura, T. (1989) Active complex between adrenodoxin reductase and adrenodoxin in the cytochrome P-450scc reduction reaction. *J. Biochem*. **105,** 601–605.
13. Hara, T., and Takeshima, M. (1994) Conclusive evidence of a quaternary cluster model for cholesterol side chain cleavage reaction catalyzed by cytochrome P-450scc. *In* Cytochrome P-450: Biochemistry, Biophysics, and Molecular Biology (Lechner, M. C., Ed.), pp. 417–420, Libbey Eurotext, Paris.
14. Ohashi, M., and Omura, T. (1978) Presence of the NADPH–cytochrome P-450 reductase system in liver and kidney mitochondria. *J. Biochem*. **83,** 249–260.
15. Sagara, Y., Hara, T., Ariyasu, Y., Ando, F., Tokunaga, N., and Horiuchi, T. (1992) Direct expression in *Escherichia coli* and characterization of bovine adrenodoxins with modified amino-terminal regions. *FEBS Lett*. **300,** 208–212.
16. Reid, E., and Williamson R. (1974) Centrifugation. *Methods Enzymol*. **31,** Part A, 713–733.
17. Kimura, T., Parcells, J. H., and Wang, H. P. (1978) Purification of adrenodoxin reductase, adrenodoxin, and cytochrome P-450 from adrenal cortex. *Methods Enzymol*. **52,** Part E, 132–142.
18. Green, N. M. (1970) Spectrophotometric determination of avidin and biotin. *Methods Enzymol*. **18,** Part A, 418–424.
19. Lowry, O. H. Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193,** 265–275.
20. Segel, I. H. (1993) *in* Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, pp. 218–220, Wiley, New York.
21. Bayer, E. A., and Wilchek, M. (1980) The use of the avidin–biotin complex as a tool in molecular biology. *Methods Biochem. Anal*. **26,** 1–45.
22. Okamura, T., John, M. E., Zuber, M. X., Simpson, E. R., and Waterman, M. R. (1985) Molecular cloning and amino acid sequence of the precursor form of bovine adrenodoxin: Evidence for a previously unidentified COOH-terminal peptide. *Proc. Natl. Acad. Sci. USA* **82,** 5705–5709.
23. Millett, F., and Geren, L. M. (1991) Chemical modification of interaction between adrenodoxin and cytochrome P450scc. *Methods Enzymol*. **206,** 49–56.
24. Hamamoto, I., Hiwatashi, A., and Ichikawa, Y. (1986) Zonal distribution of cytochromes P-450 and related enzymes of bovine adrenal cortex-quantitative assay of concentrations and total contents. *J. Biochem*. **99,** 1743–1748.
25. Müller, A., Müller, J. J., Muller, Y. A., Uhlmann, H., Bernhardt, R., and Heinemann, U. (1998) New aspects of electron transfer revealed by the crystal structure of a truncated bovine adrenodoxin, Adx(4–108). *Structure* **6,** 269–280.
26. Atsuta, Y., and Okuda, K. (1978) Isolation of rat liver mitochondrial ferredoxin and its reductase active in the 5$\beta$-cholestane-3$\alpha$, 7$\alpha$, 12$\alpha$-triol 26-hydroxylase. *J. Biol. Chem*. **253,** 4653–4658.
27. Pedersen, J. I., Ghazarian, J. G., Orme-Johnson, N. R., and Deluca, H. F. (1976) Isolation of chick renal mitochondrial ferredoxin active in the 25-hydroxyvitamin D3–1$\alpha$-hydroxylase system. *J. Biol. Chem*. **251,** 3933–3941.