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Steroid Transhydrogenase Activity of 3-Ketosteroid- Δ^1 -Dehydrogenase from *Nocardia corallina*

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3-Ketosteroid- Δ^1 -dehydrogenase from *Nocardia corallina* catalyzes transhydrogenation of 3-keto-4-ene-steroid to 3-keto-1,4-diene-steroid *e.g.*, progesterone to 1,4-androstadiene-3,17-dione. The reaction proceeded linearly at first and then soon slowed down owing to equilibration. The turnover number of this reaction was of the same magnitude as that of the dehydrogenation of 3-keto-4-ene-steroid. The pH optimum was 8.4, which is lower than that of the dehydrogenase reaction. The enzyme has a wide specificity for hydrogen acceptor steroids. The K_m' and K_{max}' values for these steroids and the values of the corresponding 3-keto-4-ene-steroids were compared. Kinetic studies of the steroid transhydrogenase reaction demonstrated a typical ping-pong mechanism. The enzyme oxidized 1,2-tritiated progesterone and transferred the tritium atoms to the reaction product, 4-androstene-3,17-dione, and water. Transhydrogenation in D_2O resulted in the incorporation of a deuterium atom into the C2-position of 4-androstene-3,17-dione. The results indicate that the enzyme catalyzes C1,C2-*trans* axial abstraction of hydrogen atoms from progesterone, transfer of the 1α -hydrogen to the C1-position of 1,4-androstadiene-3,17-dione and release of the 2β -hydrogen to water. Reaction schemes based on the experimental results are proposed. The enzyme also catalyzes the reduction of 3-keto-1,4-diene-steroids with reduced benzyl viologen.

3-Ketosteroid- Δ^1 -dehydrogenase [EC 1.3.99.4] catalyzes the oxidation of 3-keto-4-ene-steroids with the insertion of a double bond between the C1 and C2 positions. The catalytic properties of the enzyme were studied with crude extracts from microorganisms in the early 1960s (1-3). Recently, we have purified the enzyme from *Nocardia corallina* and characterized it as a flavo-protein dehydrogenase (4). The enzyme catalyzes the dehydrogenation of various kinds of 3-ketosteroids with large molecular activities. Spectral studies of this enzyme indicate that the flavin prosthetic group is reduced rapidly by dehydrogenation of the substrate and reoxidized by dyes or molecular oxygen. Under anaerobic conditions, the reduced enzyme alters gradually to the stable anionic semiquinone species at neutral pH (5). We proposed reaction schemes for desaturation of 3-ketosteroids by the dehydrogenase based on the properties of the semiquinone species (5). Since the rupture of two kinetically stable C-H bonds by this enzyme is similar to the reactions of mammalian general acyl-CoA dehydrogenases (6-9), glutaryl-CoA dehydrogenase (10) and acyl-CoA oxidase (11, 12), it is of interest to study the catalytic mechanism of 3-ketosteroid- Δ^1 -dehydrogenase. All of these dehydrogenases are flavo-dehydrogenases and eliminate two hydrogen atoms from C₁ and C₂ of CoA-derivatives of aliphatic carboxylic acids.

This paper reports the finding that 3-ketosteroid- Δ^1 -

dehydrogenase also catalyzes the transhydrogenation between 3-keto-4-ene-steroid and 3-keto-1,4-diene-steroid and the reduction of 3-keto-1,4-diene-steroid with dithionite-reduced benzyl viologen.

MATERIALS AND METHODS

Materials—*N. corallina* 3-ketosteroid- Δ^1 -dehydrogenase was prepared as previously reported (4) and stored at -80°C until use. Concentrations of the enzyme were calculated by using an ϵ_{457} value of $11.3\text{ mM}^{-1}\cdot\text{cm}^{-1}$ for enzyme-bound flavin. Steroids were purchased from Sigma, Steraloids and Nacalai Tesque. D_2O was the product of CEA-France Atomic. [$1\alpha,2\alpha(n)^3\text{H}$]Progesterone was from Amersham (tritium label distribution on the steroid was 44% on 1α , 8.5% on 1β , 40% on 2α , and 7.5% on 2β -position). Phenazine methosulfate (PMS) was from Sigma. 3-Keto-1,4-diene-steroids listed in Table II were prepared by dehydrogenation of the 3-keto-4-ene-derivatives catalyzed by 3-ketosteroid- Δ^1 -dehydrogenase on a large scale and purified by thin layer chromatography on silica gel plates (4). The purity of each prepared steroid was examined by thin layer chromatography. Concentrations of the steroids dissolved in ethanol were determined by using $\epsilon_{240} = 16.3\text{ mM}^{-1}\cdot\text{cm}^{-1}$ (13). Other chemicals were of reagent grade, obtained from commercial sources.

Methods—**Assay of 3-ketosteroid- Δ^1 -dehydrogenase**: The activity was assayed by the method using 4-androstene-3,17-dione as the substrate and PMS as the hydrogen acceptor (4).

Assay for steroid transhydrogenase activity: The standard assay solution was $100\ \mu\text{M}$ progesterone, $100\ \mu\text{M}$

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Abbreviations: PMS, phenazine methosulfate; Ches, 2-(cyclohexylamino)ethanesulfonic acid; DCIP, 2,6-dichlorophenolindophenol; progesterone, 4-pregnene-3,20-dione; 19-nortestosterone, 19-nor-4-androsten-17 β -ol-3-one; estradiol, 1,3,5(10)estratriene-3,17-diol; 11 α -hydroxyprogesterone, 4-pregnen-11 α -ol-3,20-dione.

1,4-androstadiene-3,17-dione, 50 mM Na-Ches bufer, pH 9.0, in a total volume of 1.0 ml. The reaction was started by addition of the enzyme solution. The assays were performed at 37 or 4°C and terminated by addition of 1 ml of chloroform-methanol (1 : 1, v/v). The steroids-containing chloroform phase was separated, and evaporated to dryness, and the residue was dissolved in 100 μ l of acetonitrile. The steroids were analyzed by HPLC with a linear gradient solvent system of 40–60% acetonitrile-water on an ODS column (2.5 \times 150 mm) (Chemcosorb, Chemco Scientific, Osaka) at a flow rate of 0.250 ml/min. The chromatogram was monitored at 240 nm. Under these conditions, the retention times of 1,4-androstadiene-3,17-dione, 4-androstene-3,17-dione, 1,4-pregnadiene-3,20-dione, and progesterone are 17.7, 23.0, 31.6, and 39.1 min, respectively.

A photometric assay was employed to follow dehydrogenation of 19-nortestosterone by monitoring the decrease of absorbance at 247 nm. The assay solution was 50 μ M 19-nortestosterone, 50 μ M 3-keto-1,4-diene-steroid, 50 mM Na-Ches buffer, pH 9.0, in a total volume of 2.0 ml. The reaction was started by addition of the enzyme solution and was carried out at 37°C. The decrease of absorbance at 247 nm was followed, and the initial rate of the reaction was determined. Concentrations of 19-nortestosterone were estimated by using an ϵ_{247} value of 16.2 mM⁻¹·cm⁻¹ (13).

Transhydrogenation using [$1\alpha,2\alpha(n)^3$ H]progesterone: The reaction mixture contained 100 μ M [$1\alpha,2\alpha(n)^3$ H]-progesterone (33 \times 10⁴ dpm), 100 μ M 1,4-androstadiene-3,17-dione in 1.0 ml total volume of 30 mM Tris-HCl buffer, pH 7.4. The reaction was started by addition of the enzyme solution (7 pmol). After incubation for 20 min at 30°C, the reaction was terminated by addition of 1 ml of ethyl acetate. The reaction mixture was extracted subsequently with three 3-ml aliquots of ethyl acetate, and the solvent was evaporated off under a stream of nitrogen. The dried extract was dissolved in acetonitrile and injected into an ODS column for HPLC. The chromatography was performed by using a linear gradient solvent system as described above. Aliquots of 0.3-ml fractions eluted from the column were collected and mixed with 3 ml of ACS II aqueous counting scintillant (Amersham) for determination of ³H-radioactivity.

For determination of release of tritium from the labeled progesterone into water, the same reaction was carried out in a Thunberg-type tube under reduced N₂ pressure. After incubation for 20 min at 37°C, the assay tube was immersed in a boiling water bath, and the side arm was cooled in an ice bath to recover the water from the reaction mixture. An aliquot of 0.1 ml of the water obtained in the side arm was used for determination of the radioactivity.

Steroid transhydrogenation in deuterium water: The deuterium water solutions of the reaction buffers were prepared by freeze-drying 0.03 M Tris-HCl buffer, pH 7.4, and 0.05 M Na-Ches buffer, pH 9.0, and redissolving the residue in the original volume of D₂O. The pD values of buffers were checked before use. The purified 3-ketosteroid- Δ^1 -dehydrogenase was freeze-dried and then dissolved in D₂O to the original volume. The reaction mixture contained 500 μ M progesterone, 500 μ M 1,4-androstadiene-3,17-dione, and 350 pmol of the enzyme in 1 ml of D₂O buffer. After the reaction for 40 min at 30°C, steroids were extracted with 15 ml of ethyl acetate and chromato-

graphed on an HPLC column. The fractions containing 4-androstene-3,17-dione were collected and combined. The steroid was extracted with chloroform, and the solvent was evaporated off under a stream of nitrogen. Mass spectral analysis of the steroid was performed at an ionization energy of 24 eV and an emission current of 30 μ A with a Hitachi model M-80 mass spectrometer equipped with a data processing apparatus.

Alkaline equilibration of deuterium-incorporated steroids: Deuterated 4-androstene-3,17-dione was refluxed in methanol-1% KOH mixture (2 : 1, v/v) for 2 h (3). The solution was neutralized with 1 N HCl. The steroid was extracted with ethyl acetate and purified by thin layer chromatography. The deuterium content of the product was determined by mass spectrometric analysis.

Reduction of 1,4-androstadiene-3,17-dione with reduced benzyl viologen: The reaction mixture contained 400 μ M 1,4-androstadiene-3,17-dione, 1.5 mM benzyl viologen, 7.5 mM Na-dithionite, and the enzyme (100–800 pmol) in 1 ml total volume of 0.03 M Tris-HCl buffer, pH 6.6, in a Thunberg-type tube. After evacuation, the reaction was started by mixing the dithionite solution in the side arm with the reaction mixture in the main tube and was continued for 20 min at 37°C. Analyses of the reaction mixture were performed by HPLC on an ODS column as described above.

The ¹H-NMR spectra of steroids in CDCl₃ were obtained at 400 MHz with a JEOL 400M spectrometer. Samples were analyzed in 5-mm NMR tube. Assignments of the spectra of sample steroids were performed by comparison with the spectra of the authentic compounds.

RESULTS

Steroid Transhydrogenase Activity—*N. corallina* 3-ketosteroid- Δ^1 -dehydrogenase catalyzes the dehydrogenation of several kinds of 3-ketosteroids with reduction of a hydrogen acceptor such as dyes, menadione or molecular oxygen (4). When the enzyme was incubated with 3-keto-4-ene-steroid and 3-keto-1,4-diene-steroid, the 4-ene-steroid was oxidized and the 1,4-diene-steroid was reduced. Progesterone and 1,4-androstadiene-3,17-dione were incubated, and the reaction mixtures were analyzed by TLC and HPLC. Figure 1 shows the formations of 1,4-pregnadiene-3,20-dione and 4-androstene-3,17-dione. This reaction proceeded rapidly and stoichiometrically, and depended on the amount of the enzyme (Fig. 2) and on the incubation time. Under the standard assay conditions used for the assay of the dehydrogenase activity (4) except for the replacement of PMS and DCIP with 1,4-androstadiene-3,17-dione, the reaction proceeded fast and linearly for a few minutes and then at a gradually decreasing rate. The figures indicate that the oxidation of progesterone and the reduction of 1,4-diene-steroid take place by transhydrogenation between the two steroids. Rates of this reaction with the standard assay system were the same under anaerobic and aerobic conditions, indicating that molecular oxygen does not prohibit the transhydrogenation. When the reaction was attempted with low concentrations of the enzyme at 4°C, a linear relationship was obtained between the activity and the amount of enzyme or the incubation time (Fig. 3). The result reveals the turnover number of the reaction between progesterone and 1,4-androstadiene-

Fig. 1. HPLC elution pattern of steroids after the incubation of progesterone (PG) and 1,4-androstadiene-3,17-dione (ADD) with 3-ketosteroid- Δ^1 -dehydrogenase. The enzyme, 4 pmol, was incubated with 100 μ M progesterone and 100 μ M 1,4-androstadiene-3,17-dione in 1 ml of 0.05 M Na-Ches buffer, pH 9.0, for 30 min at 30°C. The reaction was terminated by addition of 3 ml of ethyl acetate, and the extract was dried under a nitrogen stream. HPLC analysis was carried out under the conditions described in "MATERIALS AND METHODS." The identities of the steroids were confirmed by comparison of the retention times with those of the authentic compounds. Δ^1 -PG, 1,4-pregnadiene-3,20-dione; and AD, 4-androstene-3,17-dione.

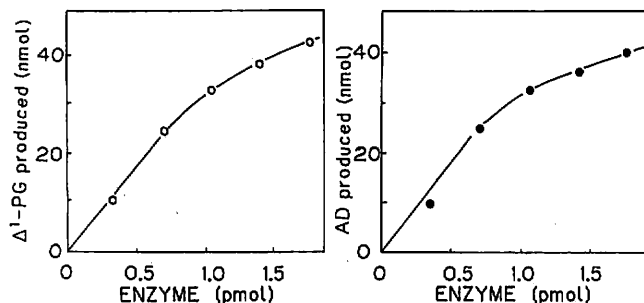
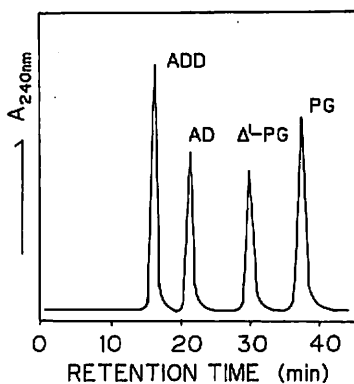


Fig. 2. Effect of the concentration of 3-ketosteroid- Δ^1 -dehydrogenase on the transhydrogenase activity. The reaction mixture contained 100 μ M progesterone and 100 μ M 1,4-androstadiene-3,17-dione in 1 ml of 0.05 M Na-Ches buffer, pH 9.0. The reaction was started by addition of the indicated amount of enzyme, and the mixture was incubated for 5 min at 37°C. Products, 1,4-pregnadiene-3,20-dione (Δ^1 -PG) and 4-androstene-3,17-dione (AD), were analyzed by HPLC.

3,17-dione to be 2,000 min^{-1} . The enzyme also catalyze the reduction of 1,4-pregnadiene-3,20-dione to progesterone with stoichiometric consumption of 4-androstene-3,17-dione. This finding indicates that the enzyme-catalyzed dehydrogenation is reversible and ends at an equilibrium state. In the progesterone/1,4-androstadiene-3,17-dione coupled reaction, the chemical equilibrium was established at 45% conversion of the substrates to the products at the optimum pH (Table I). Similar values for the equilibrium constant were obtained by starting from either side of the reaction, and ΔG_0 was estimated to be -0.7 kcal/mol based upon the constant. For the 11 α -hydroxyprogesterone/1,4-androstadiene-3,17-dione couple, the equilibration was somewhat slower and the constant was evaluated to be 3.1, which reflects the difference of K_m values (4). The enzyme catalyzed the conversion of 19-nortestosterone to estradiol with reduction of 1,4-androstadiene-3,17-dione. The reaction with 19-nortestosterone/1,4-androstadiene-3,17-dione couple yielded about 60% conversion of the substrates. Product inhibition by estradiol did not allow further conversion (4). The reverse reaction was not catalyzed by the enzyme because the product in the forward reaction is estradiol. Using the spectrophotometric assay

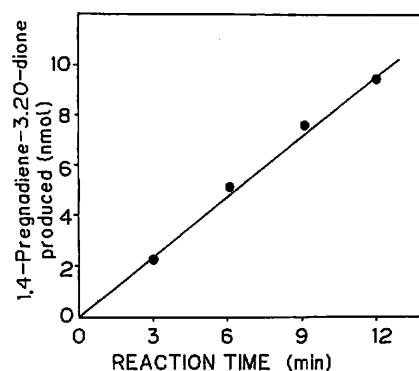


Fig. 3. Transhydrogenation with 3-ketosteroid- Δ^1 -dehydrogenase at low temperature. The reaction mixture contained 200 μ M progesterone and 200 μ M 1,4-androstadiene-3,17-dione in 2 ml of 0.03 M Tris-HCl buffer, pH 7.4. After preincubation at 4°C, the enzyme solution, 0.26 pmol in bovine serum albumin solution (1 mg/ml), was added to start the reaction. Products were assayed by HPLC. The data shown in the figure are the amounts of produced 1,4-pregnadiene-3,20-dione.

TABLE I. Equilibration in the transhydrogenation reaction catalyzed by 3-ketosteroid- Δ^1 -dehydrogenase.

Exp. A.	PG*	ADD	Δ^1 -PG	AD	K
1. Initial conc. (μ M)	105.5	102.1	0	0	0.70
Final conc. (μ M)	54.7	58.2	50.8	44.0	
2. Initial conc. (μ M)	0	0	95.3	100.7	0.68
Final conc. (μ M)	44.0	44.6	51.3	56.1	
Exp. B.	11 α -OHPG	ADD	Δ^1 -11 α -OHPG	AD	
3. Initial conc. (μ M)	83.8	102.1	0	0	3.10
Final conc. (μ M)	26.0	42.6	57.8	59.5	

*PG, progesterone; ADD, 1,4-androstadiene-3,17-dione; Δ^1 -PG, 1,4-pregnadiene-3,20-dione; AD, 4-androstene-3,17-dione; 11 α -OHPG, 11 α -hydroxyprogesterone; Δ^1 -11 α -OHPG, 11 α -hydroxy-1,4-pregnadiene-3,20-dione.

TABLE II. Apparent kinetic constants for 3-keto-1,4-diene-steroids.

Steroid	K_m' (μ M)	V_{max}' (mol/min/mol FAD)
1,4-Androstadiene-3,17-dione (Δ^1 -Androstenedione)	4	2,436
1,4-Pregnadien-21-ol-3,20-dione (11-Deoxy- Δ^1 -corticosterone)	21	1,870
1,4-Pregnadiene-17 α ,21-diol-3,11,20-trione (Δ^1 -Cortisone)	9.1	1,740
1,4-Pregnadiene-17 α ,21-diol-3,20-dione (11-Deoxy-17 α -hydroxy- Δ^1 -corticosterone)	2.7	1,680
1,4-Androstadiene-3,11,17-trione (Δ^1 -Adrenosterone)	3.8	1,310
1,4-Pregnadien-11 α -ol-3,20-dione (11 α -Hydroxy- Δ^1 -progesterone)	17	305
1,4-Pregnadiene-11 β ,21-diol-3,20-dione (Δ^1 -Corticosterone)	18	222
1,4-Androstadien-11 β -ol-3,17-dione (11 β -Hydroxy-1,4-androstadiene-3,17-dione)	6.7	146
1,4-Pregnadiene-3,20-dione-11 β ,17 α ,21-triol (Δ^1 -Hydrocortisone)	13	136

method, K_m' values and V_{max}' values for eight 1,4-diene steroids, whose corresponding 4-ene-compounds are substrates for the dehydrogenase (4), were determined in the transhydrogenase reaction as shown in Table II.

Fig. 4. A: Steady-state kinetics of steroid transhydrogenation with 3-ketosteroid- Δ^1 -dehydrogenase. The reaction mixture contained various concentrations of 1,4-androstadiene-3,17-dione with fixed concentrations of progesterone [(■) 2.2, (□) 4.4, (●) 6.7, (○) 8.8 μ M] in 2.0 ml 0.03 M Tris-HCl buffer, pH 8.2. The enzyme was added at the start of the reaction. B: Secondary plot of the reciprocal of V_{max} vs. the reciprocal of progesterone concentration.

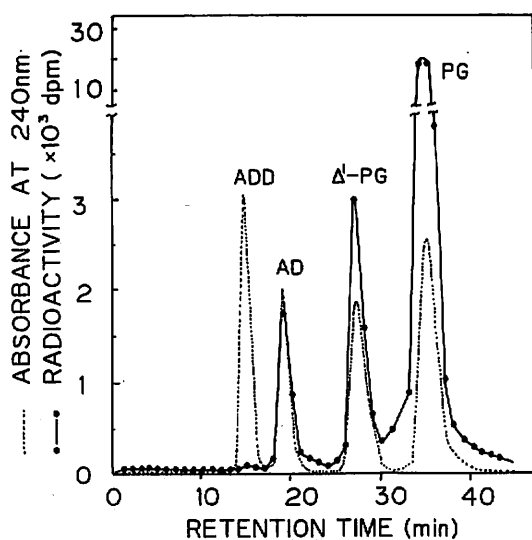
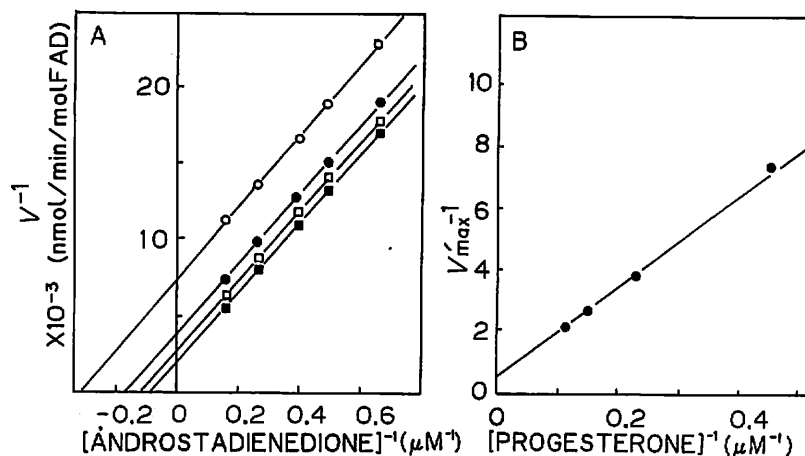


Fig. 5. Distribution of tritium in steroids after the transhydrogenase reaction using [$1\alpha,2\alpha(n)$ - 3 H]progesterone. The reaction conditions are given in "MATERIALS AND METHODS." AD, 4-androstene-3,17-dione; ADD, 1,4-androstadiene-3,17-dione; PG, progesterone; and Δ^1 -PG, 1,4-pregnadiene-3,20-dione.

The optimum pH of the transhydrogenation was 8.2 as determined by the HPLC method with progesterone and 1,4-androstadiene-3,17-dione and also by the spectrophotometric method with 19-nortestosterone. This value is lower by 2 pH units than that of the dehydrogenase activity (4).

Kinetics of Steroid Transhydrogenation—Figure 4A illustrates the effect of varying the concentration of 1,4-androstadiene-3,17-dione on the rate of transhydrogenation at various fixed concentrations of progesterone. A set of parallel lines was obtained in the double-reciprocal plot, and the following kinetic values were determined by secondary plots of the intercepts vs. the reciprocal of concentrations of progesterone (Fig. 4B): K_m for progesterone = 25 μ M, K_m for 1,4-androstadiene-3,17-dione = 44 μ M, and $V_{max}/e_0 = 2,000 \text{ min}^{-1}$. Since the slopes in the figure are essentially parallel, the reaction proceeds through a ping-pong mechanism (14).

Transhydrogenation of [$1\alpha,2\alpha(n)$ - 3 H]Progesterone—

TABLE III. Distribution of tritium after the transhydrogenase reaction with [$1\alpha,2\alpha(n)$ - 3 H]progesterone and 1,4-androstadiene-3,17-dione. Experimental conditions are described in "MATERIALS AND METHODS."

Compound	Radioactivity		Distribution of 3 H among products (%)
	(dpm)	(%)	
Progesterone	261,452	82	—
Δ^1 -Progesterone	36,029	11.3	50.8
Androstenedione	21,362	6.7	30.1
Water	13,580	4.1	19.1

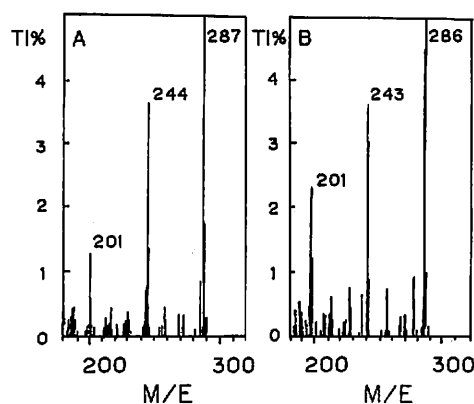


Fig. 6. Mass spectra of 4-androstene-3,17-dione (A) prepared from 1,4-androstadiene-3,17-dione by transhydrogenation in D_2O . (B) After alkaline equilibration of (A).

When [$1\alpha,2\alpha(n)$ - 3 H]progesterone was oxidized with the enzyme in the presence of 1,4-androstadiene-3,17-dione, tritium was incorporated into 4-androstene-3,17-dione, 1,4-pregnadiene-3,20-dione, and also water (Fig. 5 and Table II). Incorporations of tritium into the hydrogen-acceptor steroid and into water were not observed in the absence of the enzyme. The data in Table III show the distributions of tritium incorporated into steroids and water after the incubation. It appears that hydrogen atoms are eliminated at the C1- and C2-positions of progesterone are eliminated and transferred directly to the 1,4-androstadiene-3,17-dione molecule and to water. When the tritium-incorporated 4-androstene-3,17-dione was subjected to alkaline equilibration, the specific activity of tritium did not change;

i.e. the tritium is located at the C1-position of the steroid.

Transhydrogenation in D_2O —The transhydrogenation from progesterone to 1,4-androstadiene-3,17-dione was performed for 40 min at 30°C in 0.03 M Tris-DCI buffer (pD 7.4) and 0.05 M NaOD-Ches buffer (pD 9). The reduced product was isolated by HPLC and examined by mass spectral analyses. The results (shown in Fig. 6A) indicate that the value of molecular ion of the steroid ($M^+ = 286$) is altered to $M^+ = 287$ by incorporation of one atom of deuterium from D_2O . The NMR spectrum in $CDCl_3$ supported the results of mass analysis. The figure also shows the mass spectrum of the deuterated steroid after alkaline equilibration (Fig. 6B). This treatment alters the value of the molecular ion to 286, and indicates that the deuterium atom is located at the C2-position of the steroid. The deuterium incorporation was not observed in the absence of the enzyme.

Reduction of 1,4-Androstadiene-3,17-Dione with Reduced Benzyl Viologen—3-Ketosteroid- Δ^1 -dehydrogenase catalyzed the reduction of 1,4-androstadiene-3,17-dione in the presence of dithionite and benzyl viologen under anaerobic conditions. The reaction proceeded linearly up to 800 pmol of the enzyme and up to 20 min incubation time. The turnover number was 5.4 min^{-1} : this value is very much lower than those of the dehydrogenation and transhydrogenation. The optimum pH of this reductase reaction was 6.6.

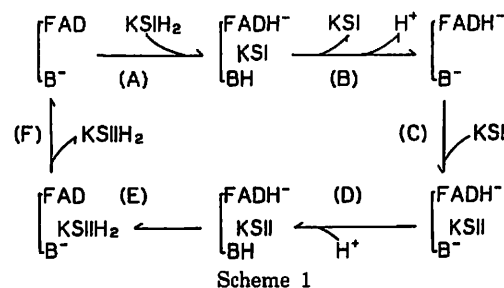
DISCUSSION

The purified 3-ketosteroid- Δ^1 -dehydrogenase catalyzes the abstraction of C1 and C2-hydrogens of 3-ketosteroid with the reduction of an artificial hydrogen acceptor at a high reaction rate. In the present study, we found that the enzyme exhibits unusual catalytic activity for steroid transhydrogenation under steady-state conditions, that is, the oxidation of 3-keto-4-ene-steroid coupled stoichiometrically with the reduction of 3-keto-1,4-diene-steroid. The turnover number of the reaction is of the same order of magnitude as that of the dehydrogenation (4). This could be explained by the fact that the reduced FAD of the enzyme transfers the hydrogen rapidly to 1,4-diene-steroid in the reverse reaction.

The transhydrogenation catalyzed by this enzyme showed a wide specificity for substrates, resembling that of the dehydrogenation of 3-keto-4-ene-steroids. Comparisons of K_m values and also V_{max} values for 4-ene-steroids and for 1,4-diene-steroids reveal that the affinities and the reactivities for the hydrogen-accepting steroids are similar to those for the corresponding 4-ene-steroids. The influence of the 11β -hydroxyl group seen in the dehydrogenation (4) was also observed in transhydrogenation with 11β -hydroxyl-3-keto-1,4-diene steroids. These facts suggest that hydrogen-donor steroid and hydrogen-acceptor steroid molecules are bound at the same site of the enzyme.

Kinetic studies of the reaction demonstrate that the steroid transhydrogenation proceeds through a ping-pong mechanism, in which the hydrogen-donating steroid reacts first to reduce the flavin of the enzyme, then dissociates, making way for the hydrogen-accepting steroid to react at the same site of the enzyme molecule. A ternary complex of the enzyme and 3-ketosteroid molecules does not form during the reaction. The optimum pH of transhydrogena-

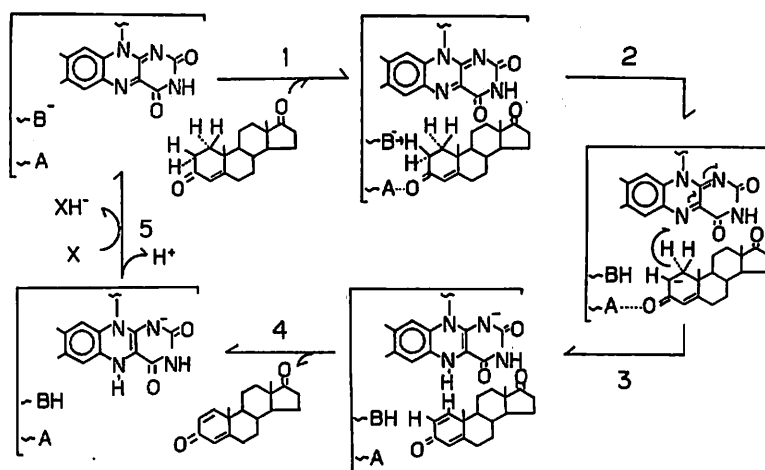
tion (pH 8.2) is lower than that of dehydrogenation (pH 10) (4), and is higher than that of reduction of 3-keto-1,4-diene-steroid with reduced dye (pH 6.6). It is interesting to note that general fatty acyl-CoA dehydrogenase, which is a flavoenzyme catalyzing β -oxidation of the substrates, also exhibits a transhydrogenation reaction at a slow rate (15, 16). In these enzyme, the reduced enzyme-product complex, probably product charge-transfer complex, dissociates the product rapidly and then transfers the reducing equivalent to the acceptor. These facts may be summarized by the equations shown in Scheme 1.



In the scheme $KSIH_2$ is a 3-keto-4-ene-steroid, and KSI is the corresponding 3-keto-1,4-diene-steroid. $KSIH_2$ and KSI are the other 3-ketosteroids. Reaction (A) is the dehydrogenation and reaction (B) is the dissociation of the product and the release of a proton from an amino acid residue (BH). Similarly, reactions (C), (D), (E), and (F) proceed. Dissociation of BH to B^- and H^+ promotes the proton exchange between steroid and water.

The experiments with $[1\alpha, 2\alpha(n)-^3H]$ progesterone revealed the stereochemistry of the dehydrogenation. The incorporations of tritium into the C1-position of 1,4-androstadiene-3,17-dione and water from the labeled progesterone and the incorporation of deuterium into the C2-position of 1,4-androstadiene-3,17-dione by the reaction in D_2O revealed the stereochemical configurations of the abstracted hydrogen atoms. The distributions of tritium in both product steroids exclude the possibility of *cis*-abstraction, and hydrogens at the 1α and 2β -positions are eliminated from progesterone; the former hydrogen is transferred to the C1-position of 1,4-androstadiene-3,17-dione, and the latter hydrogen is removed to water through a nucleophilic amino acid residue. The C2-hydrogen in the produced 4-androstene-3,17-dione molecule is derived from the reaction medium. As described in a previous paper (5), the flavin semiquinone species of 3-ketosteroid- Δ^1 -dehydrogenase is not involved in the dehydrogenation. This *trans*-axial elimination was also observed in fermentation experiments using deuterated steroids (2). It should be emphasized that these mechanisms are substantially the same as those of the reactions performed with general acyl-CoA dehydrogenases (17-20). In contrast, the aromatization of 4-androstene-3,17-dione to estrone by placental aromatase, a kind of P-450 enzyme, proceeds with *cis*-abstraction of hydrogen atoms from the C1 and C2 positions (21, 22).

As indicated in the previous paper (4), the enzyme absolutely requires the carbonyl group at the C3-position of the substrate steroid. The carbonyl oxygen might interact strongly with an electrophilic residue of the enzyme protein, stimulating the rupture of the C2(β)-H bond. The hydrogen might be transferred by a protein base to a



Scheme 2

nucleophilic residue as a proton. Therefore, a fast exchange of the hydrogen can occur in the reaction. The substrate steroid forms a carbanion intermediate, and the C1(α)-H bond is ruptured concomitantly to form a hydride ion, which is transferred to the N(5)-position of the oxidized flavin. The mechanism can thus be formulated as shown in Scheme 2. The residue A is an electrophile, and the residue B is a nucleophile. In general acyl-CoA dehydrogenase, the residue B is reported to be a glutamate residue (23). The above discussion is supported by the fact that the enzyme catalyzes exchange of the hydrogen at the C2-position of 4-androstene-3,17-dione with hydrogen ion upon anaerobic incubation, when about 80% of the flavin was in the reduced state (our unpublished data). At the latter half-reactions of the transhydrogenation, reduction of diene-steroid occurs with the reversed sequence of these reactions, and N(5)-H does not exchange but transfers to the C1-position of the acceptor steroid. Studies on isotope effects by using deuterated steroids could provide more information about the stereochemical aspects of the transhydrogenation and also the dehydrogenation of 3-ketosteroids by the 3-ketosteroid- Δ^1 -dehydrogenase. Kinetic studies of the initial phase of the reactions are also required to examine this interesting reaction in more detail.

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