3-keto-5α-steroid-Δ4-dehydrogenase from Nocardia corallina: Purification and characterization

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3-Keto-5α-Steroid-Δ⁴-Dehydrogenase from *Nocardia corallina*: Purification and Characterization

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The inducible 3-keto-5α-steroid-Δ⁴-dehydrogenase of *Nocardia corallina* was purified to homogeneity using affinity chromatography on 19-nortestosterone-17-acetoxyaminoethyl Sepharose 4B. SDS-polyacrylamide gel electrophoresis, gel filtration and spectral analysis of flavin suggest that the purified dehydrogenase is a monomeric protein of M_r 60,000 containing one flavin. It has a typical absorption spectrum of flavoprotein with maxima at 457, 375, and 277 nm. The values shifted to 470 and 395 nm on binding of 19-nortestosterone. The enzyme catalyzed the dehydrogenation of 3-keto-5α-steroid at the 4- and 5-position, *e.g.* the conversion of 5α-androst-1-ene-3,17-dione to 1,4-androstadiene-3,17-dione with the reduction of phenazine methosulfate. The substrate 3-ketosteroid has essentially the 5α-configuration. The enzyme did not reduce potassium ferricyanide but did reduce cytochrome *c* at a moderate rate, and exhibited only a weak steroid oxidase activity. Stereochemical study demonstrated that the enzyme abstracts the 4β, 5α-hydrogens of the substrate as a hydrogen ion through a protein-based reaction and as a hydride ion by transfer to FAD, respectively. The enzyme oxidizes a wide variety of 3-keto-5α-steroids but not 3β-hydroxysteroid. The dehydrogenase also catalyzed steroid transhydrogenation between 3-keto-5α-steroid and 3-keto-1,4-diene-steroid. The properties of this enzyme are compared with those of 3-keto-steroid-Δ¹-dehydrogenase.

Introduction of a double bond in the A-ring of 3-ketosteroids is an important step in microbial metabolism of steroids. 3-Ketosteroid-Δ¹-dehydrogenase is an FAD-containing enzyme catalyzing the desaturation of steroids by abstraction of C1α- and C2β-hydrogens from the thermodynamically stable C-H bonds of the substrate steroid. The enzymes from *Nocardia corallina* and *Nocardia opatica* were purified to homogeneity (*1-3*), and their spectrophotometric and enzymic properties were characterized (*1, 2*). The enzyme is a typical flavo-dehydrogenase and forms a stable semiquinone under various experimental conditions (*2*). Studies on the steroid transhydrogenase reactions disclosed the mechanism of cleavage of C-H bonds of the steroid nucleus (*4, 5*). In the course of these studies, we found an enzyme which catalyzes dehydrogenation of the C4-C5 position of 3-ketosteroids in partially purified enzyme preparations; it is 3-keto-5α-steroid-Δ⁴-dehydrogenase. The enzyme from *Pseudomonas testosteroni* was described by Levy and Talalay three decades ago (*6*), but its characteristics were not reported. The activity of the Δ⁴-dehydrogenase is much lower than that of the Δ¹-dehydrogenase in a crude extract of *N. corallina*, and its isolation was difficult by general chromatographic procedures.

In the present study, we synthesized a new affinity gel containing 17-acetoxy-19-nortestosterone as the ligand and applied it to the purification of 3-keto-5α-steroid-Δ⁴-dehydrogenase from *N. corallina*. We were able to purify the enzyme to homogeneity and characterized it as a flavodehydrogenase having one FAD molecule as its prosthetic group. This paper describes the isolation, and the molecular and enzymic properties of this enzyme, in comparison with those of the Δ¹-dehydrogenase.

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Abbreviations: Ches, 2-(cyclohexylamine)ethanesulfonic acid; DTT, dithiothreitol; DCIP, 2,6-dichlorophenol indophenol; PMS, phenazine methosulfate.

MATERIALS AND METHODS

Materials—5α-Androst-1-ene-3,17-dione, 5β-androst-1-ene-3,17-dione, 19-nortestosterone, androsta-1,4-diene-3,17-dione, and 6α-hydroxyandrost-4-ene-3,17-dione were purchased from Sigma Chemical and used without further purification. 3-Keto-1-ene-steroids listed in Table II were prepared as described below. Other chemicals were obtained from commercial sources as described (*1*).

Culture of *Nocardia corallina* and preparation of the cell extracts were carried out as described (*1*).

Synthesis of 19-Nortestosterone-Sepharose Affinity Gel—17β-Bromoacetoxy-19-nortestosterone was synthesized by the procedure described by Holmes and Smith (*7*). Activation of Sepharose 4B with BrCN and its reaction with ethylenediamine were carried out by the method of Lowe (*8*). The steroid ligand was coupled to the gel by the reported method (*9*). The concentration of immobilized steroid was estimated to be about 1 μg per g packed gel.

Purification of 3-Keto-5α-Steroid-Δ⁴-Dehydrogenase—All manipulations were carried out at 4°C. Tris-HCl (0.03 M) buffer, pH 7.4, containing 10^{-4} M EDTA was used as the standard buffer, and is termed "Tris-HCl buffer." Chromatographic procedures with the first DEAE-cellulose column and the second phenyl-Sepharose column were the same as those used for the purification of 3-ketosteroid-

Δ^1-dehydrogenase (*1*). The eluted fractions were combined and dialyzed against 3 liters of Tris-HCl buffer for 18 h and charged on an affinity gel column (5.5×7.5 cm) equilibrated with the same buffer. The column was washed with 500 ml of 0.2 M KCl-Tris HCl buffer, and the enzyme was eluted with the buffer containing 200 μM 19-nortestosterone at a flow rate of 38 ml/h. The active fractions were combined and concentrated by the Centriflo method. The purified enzyme was stored at −80°C until use.

Assay of 3-Keto-5α-Steroid-Δ^4-Dehydrogenase—Dehydrogenase activities were measured spectrophotometrically at 20°C by monitoring enzyme-stimulated reduction of 2,6-dichlorophenol indophenol accompanying substrate turnover (*1*). The reaction mixture contained 100 μM DCIP, 100 μM PMS, and an appropriate amount of enzyme in 0.5 ml of 0.05 M Ches-Na buffer, pH 9.0. The reaction was started by the addition of 100 nmol of 5α-androst-1-ene-3,17-dione in 10 μl of propyleneglycol.

The unit of activity is defined as that amount of enzyme which reduces 1.0 μmol of DCIP per min under the conditions of the routine assay.

Analysis of steroids were performed by HPLC on an ODS column (*1*). Protein was determined by the method of Lowry *et al.* (*10*) with bovine serum albumin as a standard. Analyses of flavin were performed by the methods previously described (*1, 11*). Gel electrophoresis of the purified enzyme was carried out by the reported method (*12*), and the activity staining was performed by using 5α-androst-1-ene-3,17-dione as a substrate (*1*). Isoelectric-focusing gel electrophoresis was performed on a 5% polyacrylamide gel slab containing 2.5% Ampholine (pH 3.5-10 or 2.5-6) with standard marker proteins at 4°C.

Preparation of 3-Keto-1-Ene-5α-Steroid—Steroids listed in Table II were prepared by reduction of 3-keto-4-ene-steroids in the presence of a palladium catalyst with hydrogen followed by dehydrogenation with 3-ketosteroid-Δ^1-dehydrogenase. The corresponding steroid (20 mg) was dissolved in 2 ml of dioxane and was reduced under a hydrogen atmosphere in the presence of 2 mg of 10% palladium-on-carbon catalyst for 5 h (*13*). After removal of the catalyst, dioxane was evaporated off, and the residual steroid was dissolved in 1 ml of ethanol and purified by TLC on silica gel plates. The reduced steroid was dissolved in 5 ml of propyleneglycol and was added to the reaction mixture containing 200 μM PMS and about 0.5 unit of 3-ketosteroid-Δ^1-dehydrogenase in 50 ml of Tris-HCl buffer. After incubation for 3 h at 37°C, the steroid was extracted from the reaction medium into ethyl acetate and purified by TLC [with benzene/ethyl acetate/methanol, 66 : 33 : 1 (v/v), or chloroform/acetone, 9 : 1 (v/v)]. Each purified steroid was dissolved in propyleneglycol and the concentration was determined by the spectral method (*4*).

Preparation and Reaction of 4α,5α-Deuterio-Androst-1-Ene-3,17-Dione—Androst-4-ene-3,17-dione was reduced to a 5α and 5β mixture of 4,5-deuterio-androstane-3,17-dione with deuterium gas in the presence of a palladium catalyst (*13*). The mixture was then dehydrogenated with 3-ketosteroid-Δ^1-dehydrogenase as described above, and the product was isolated by TLC [with benzene/ethyl acetate/methanol, 66 : 33 : 1 (v/v)]. The identity of the obtained steroid was confirmed by mass spectrometry (*4*). The deuterium-labeled steroid was dehydrogenated with 3-keto-5α-steroid-Δ^4-dehydrogenase in the presence of 200 μM PMS for 3 h at 37°C. The reaction product was purified by HPLC and analyzed by mass spectrometry (*4*).

RESULTS

Purification of 3-Keto-5α-Steroid-Δ^4-Dehydrogenase—A typical protocol of the purification procedures described under "MATERIALS AND METHODS" and summarized in Table I resulted in an apparently homogeneous preparation of 3-keto-5α-steroid-Δ^4-dehydrogenase with a specific activity of 49.1 μmol/min/mg protein. 19-Nortestosterone-17-acetate-ligated affinity gel was synthesized and used for separation of the Δ^4-dehydrogenase from 3-ketosteroid-Δ^1-dehydrogenase in the enzyme preparation eluted from the phenyl-Sepharose column (Step 2) as shown in Fig. 1. The Δ^1-dehydrogenase bound weakly, while the Δ^4-dehydrogenase bound tightly to the gel. After washing of the gel column, the Δ^4-enzyme was released effectively in the presence of 200 μM 19-nortestosterone or androsta-1,4-diene-3,17-dione. The table shows that the Δ^4-enzyme was obtained with 35% yield and 1,403-fold purification. In the purification, a large decrease of the enzyme activity was observed in the step of DEAE-cellulose column chromatography.

Gel electrophoretic analysis of the purified enzyme in the native and denatured forms revealed a single protein band (Fig. 2). The native enzyme showed a protein stain (A) at the same position as the activity stain (B). The molecular size of the Δ^4-dehydrogenase was estimated to be 60 kDa, which is smaller than that of the Δ^1-dehydrogenase (*1*) (Fig. 2C). The results show that the two dehydrogenases can not be separated by gel-filtration on an Ultrogel ACA44 column and this indicates that the Δ^4-enzyme is also a monomeric protein, like the Δ^1-enzyme (*1*). The mobility of the Δ^4-dehydrogenase on 11% polyacrylamide slab gel at pH 7.4 is R_f=0.31, whereas that of the Δ^1-dehydrogenase is 0.58. The isoelectric point of this enzyme is 4.3, which is higher than that of the Δ^1-enzyme (*1*).

Absorption Spectrum of 3-Keto-5α-Steroid-Δ^4-Dehydrogenase—The absorption spectrum of purified Δ^4-dehydrogenase is that of a typical flavo-protein with maxima at 470, 395, 277 nm (Fig. 3, ·····). Comparisons of this spectrum with those of the purified Δ^1-enzyme and its androsta-1,4-diene-3,17-dione-bound form indicated that the iso-

TABLE I. **Summary of the purification of 3-keto-5α-steroid-Δ^4-dehydrogenase from *Nocardia corallina*.** Purification was carried out from 370 g of the frozen cells. The activity was measured by the routine assay method described in the text.

	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification yield factor (%)	
Crude extract	3,500	39,550	1,388	0.035	100	1
DEAE-cellulose	1,200	1,944	514	0.264	37	7.5
Phenyl-Sepharose	108	538	575	1.07	41	30.6
Affinity gel	115	9.8	481	49.1	35	1,403

lated Δ⁴-enzyme is a complex form with a steroid (2). The enzyme preparation after dialysis and gel-filtration on a Sephadex G-25 column showed an absorption spectrum with maxima at 457 and 375 nm (Fig. 3, ——). The flavin prosthetic group was identified as FAD by a fluorospectrometric method (11) and by thin layer chromatography in two solvent systems (1). The FAD content is 1 mol per 60,000 g of protein.

Enzyme Activity—3-Keto-5α-Steroid-Δ⁴-Dehydrogenase catalyzes the dehydrogenation of 5α-androst-1-ene-3,17-dione to androsta-1,4-diene-3,17-dione under the standard assay conditions. The product was identified by HPLC on an ODS column (Fig. 4) and thin layer chromatography on silica gel GF-274 plates. Amounts of formed

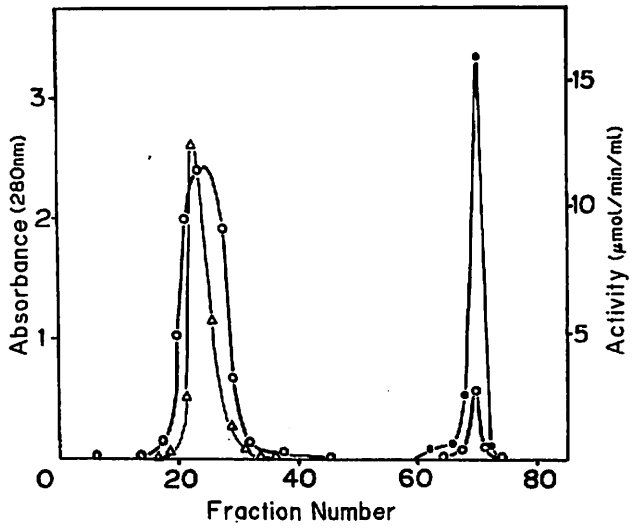


Fig. 1. **Affinity chromatography of 3-keto-5α-steroid-Δ⁴-dehydrogenase on a 19-nortestosterone-17-acetate-Sepharose column.** The enzyme preparation eluted from the phenyl-Sepharose column in Step 2 was applied to a column (5.5×7.5 cm) equilibrated with Tris-HCl buffer, pH 7.4. The column was washed with 500 ml of Tris-HCl buffer and then eluted with Tris-HCl buffer containing 200 μM 19-nortestosterone. The flow rate was 38 ml/h. Fractions of 12.8 ml were collected and assayed for protein (○), for the Δ⁴-dehydrogenase (●), and Δ¹-dehydrogenase (△) activities.

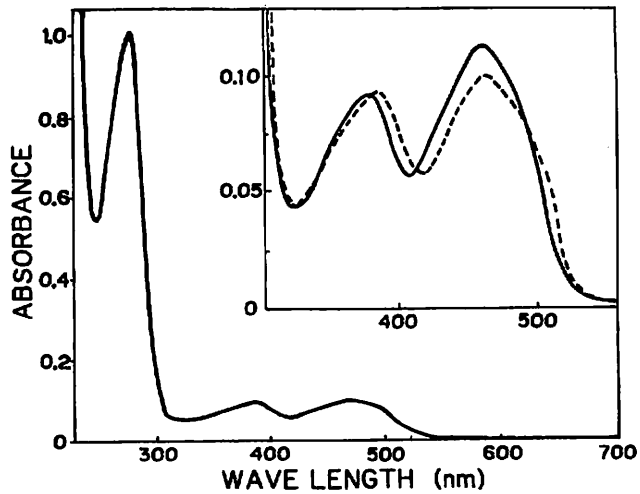


Fig. 3. **Absorption spectrum of the purified 3-keto-5α-steroid-Δ⁴-dehydrogenase.** The concentration of the enzyme was 164 μM in Tris-HCl buffer, pH 7.4. The purified enzyme (·····) and that after the gel filtration to remove 19-nortestosterone (——).

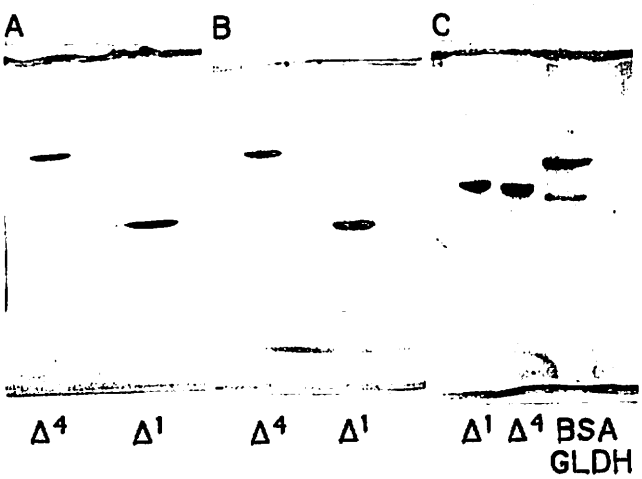


Fig. 2. **Polyacrylamide slab gel electrophoresis of the purified 3-keto-5α-steroid-Δ⁴-dehydrogenase.** Native polyacrylamide gel electrophoresis (A and B): Purified enzyme (1-10 μg) was subjected to electrophoresis on native gels of 11% total acrylamide. Gel slabs were stained for protein with Coomassie Blue (A), and for the enzyme activity with 5α-androst-1-ene-3,17-dione, PMS and nitro blue tetrazolium chloride (B). SDS-polyacrylamide gel electrophoresis of purified 3-keto-steroid-Δ¹-dehydrogenase (Δ¹), 3-keto-5α-steroid-Δ⁴-dehydrogenase (Δ⁴), and molecular weight standard proteins [bovine serum albumin (BSA) and liver glutamate dehydrogenase (GLDH)] on 9% acrylamide gel containing 0.1% sodium dodecyl sulfate. The gel slab was stained with Coomassie Blue.

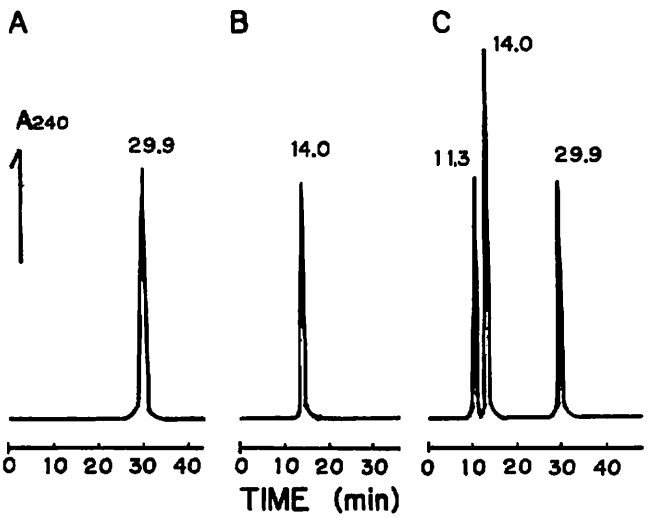


Fig. 4. **HPLC-elution pattern of steroids after incubation with 3-keto-5α-steroid-Δ⁴-dehydrogenase.** (A) Authentic 5α-androst-1-ene-3,17-dione, (B) authentic androst-1,4-diene-3,17-dione, and (C) the incubated steroids. The enzyme (9 nM) was incubated with 200 μM PMS, 200 μM 5α-androst-1-ene-3,17-dione in 0.5 ml of 50 mM Ches-Na buffer, pH 9.0, for 3 min at 34°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 reported (1).

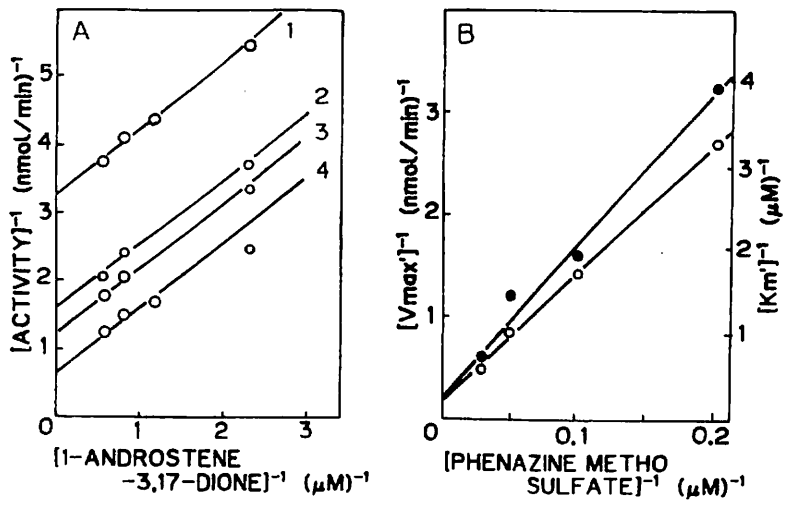


Fig. 5. **Steady-state kinetic analysis of 3-keto-5α-steroid-Δ^1-dehydrogenase.** (A) Variable concentrations of 5α-androst-1-ene-3,17-dione (0.43-1.7 μM) at fixed concentrations of PMS of 5, 10, 20, and 30 μM for lines 1-4, respectively, 67 μM DCIP, an appropriate amount of enzyme, and 0.05 M Ches-Na buffer, pH 9.0, in a total volume of 3.0 ml were used. Other conditions were the same as in the routine assay methods. (B) Secondary plots of y-intercepts (●) and K_m^{-1} (○) from A.

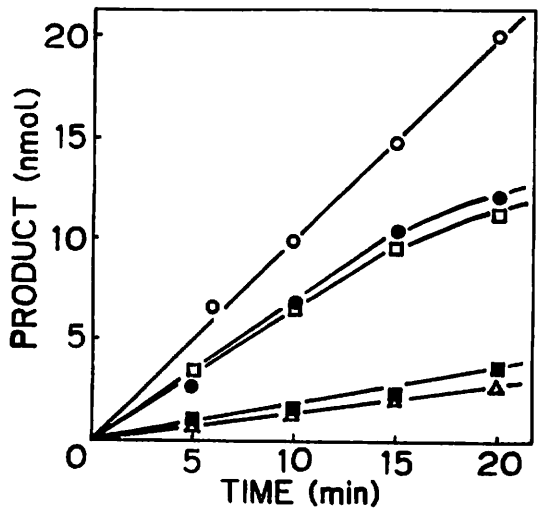


Fig. 6. **Transhydrogenation between 5α-androst-1-ene-3,17-dione and 3-keto-4-ene-steroid by 3-keto-5α-steroid-Δ^1-dehydrogenase.** The reaction mixture contained 10 μM 5α-androst-1-ene-3,17-dione and 10 μM 3-keto-4-ene-steroid in 2.0 ml of 50 mM Ches-Na buffer, pH 9.0. The reaction was started by addition of 12 nM 3-keto-5α-steroid-Δ^1-dehydrogenase and continued for the indicated period at 34°C. The reaction products were analyzed by HPLC. Hydrogen acceptor steroid; 5α-androst-4-ene-3,17-dione (○), 5α-androst-4-ene-3,11-17-trione (●), 5α-androst-4-en-6β-ol-3,17-dione (□), 5α-pregnan-11α-ol-3,20-dione (■), and 5α-androst-4-en-11β-ol-3,17-dione (△).

TABLE II. **Substrate specificity of 3-keto-5α-steroid-Δ^1-dehydrogenase.**

Substrate steroid	TN (min^{-1})	K_m (μM)
		($\times 10^3$)
5α-Androst-1-en-11β-ol-3,17-dione	0.39	66.7
5α-Pregn-1-en-11α-ol-3,20-dione	0.25	37.0
5α-Androst-1-ene-3,11,17-trione	1.61	35.7
5α-Pregn-1-ene-17α,21-diol-3,11-20-trione	2.55	83.3
5α-Androst-1-en-17β-ol-3-one	7.65	52.6
5α-Androst-1-ene-3,17-dione	11.8	4.3

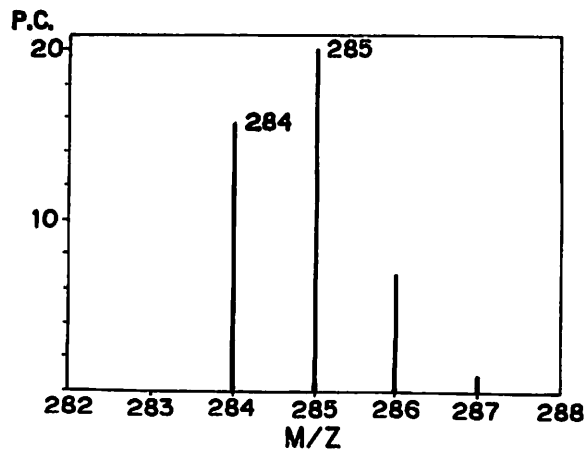
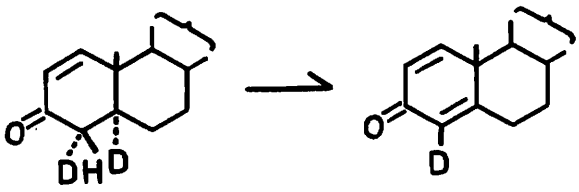


Fig. 7. **Mass spectrum of the product formed from [4α,5α-^{2}H]-androst-1-ene-3,17-dione by 3-keto-5α-steroid-Δ^1-dehydrogenase.** [4α,5α-^{2}H]Androst-1-ene-3,17-dione (120 μM) was dehydrogenated with the enzyme in 50 ml of 0.05 M Ches-Na buffer, pH 9.0, in the presence of 200 μM PMS for 3 h at 37°C. The reaction product was extracted into ethyl acetate and purified by HPLC. The figure shows part of the mass spectrum of the product.

4-ene-steroid and reduced 2,6-dichloroindophenol were in 1 : 1 stoichiometry. The reaction proceeded linearly with up to 43 pmol of enzyme in terms of the initial rate, and for up to 10 min with 0.6 pmol of enzyme under the same conditions. The purified enzyme is as stable as 3-keto-steroid-Δ^1-dehydrogenase (*1*). The pH optimum of dehydrogenation using the routine assay system is 9.5. At this pH, the maximum activity is $TN = 2.7 \times 10^3$ min^{-1} and K_m for 5α-androst-1-ene-3,17-dione is 4.4 μM (Fig. 5) and that for phenazine methosulfate is 62.5 μM. Just as in the case of 3-ketosteroid-Δ^1-dehydrogenase (*1*), the figure indicates that the enzyme reaction proceeds through a ping-pong mechanism. The steroid oxidase activity which was observed with 3-ketosteroid-Δ^1-dehydrogenase (*1*), is very low ($TN = 2.0$ min^{-1}) at pH 9.0. Potassium ferricyanide did not act as an electron acceptor, but bovine cytochrome *c* was reduced by the enzyme at a rate of 600 min^{-1} and the reaction was stimulated by the addition of menadione ($TN = 718$ min^{-1}).



Scheme 1

Transhydrogenase activity of the enzyme is observed between 3-keto-1-ene-5α-steroid and 3-keto-1,4-diene-steroid; with 5α-androst-1-ene-3,17-dione and pregna-1,4-diene-3,20-dione, the enzyme catalyzed the formation of androsta-1,4-diene-3,17-dione and pregn-1-ene-3,20-dione with the turnover number of 10 min^{-1}. Using 5α-androst-1-ene-3,17-dione as a hydrogen donor, some 4-ene-steroids were tested as hydrogen acceptors (Fig. 6). 6α-Hydroxy-androst-4-ene-3,17-dione and 11β-hydroxy-androst-4-ene-3,17-dione showed about 70 and 17% of the activity of androst-4-ene-3,17-dione, respectively.

Substrate Specificity—The enzyme oxidizes 5α-androst-1-ene-3,17-dione and 5α-androstane-3,17-dione, but does not completely oxidize 5β-androstane-3,17-dione. The enzyme has substrate specificity for 3-ketosteroids having the 5α-configuration. The corresponding 5β-steroid did not inhibit the dehydrogenation of 3-keto-5α-steroid. The reactivities of the enzyme for substrate steroids (Table II) indicate that the enzyme has a wide specificity for 3-keto-5α-steroids. 3-Keto-1-ene-steroids are better substrates than the corresponding 1,2-saturated steroids. 11-Keto-steroid is better than 11-hydroxysteroid. The reactivity is similar to that of Δ¹-dehydrogenase (*1*).

Stereochemistry of the Dehydrogenation—Since the enzyme has substrate specificity for 3-keto-5α-steroid as described above, it is clear that 5α-hydrogen is abstracted by the enzyme. Determination of stereospecificity for hydrogen at the 4-position was made by using [4α,5α-^{2}H]-androst-1-ene-3,17-dione. After incubation, the reaction product was isolated and analyzed by mass spectrometry (Fig. 7). The fact that the molecular ion, M^+, has a mass of 285 indicates that 4α-deuterium of the substrate remains in the reaction product, and the reaction proceeds with 4β, 5α-elimination of hydrogen atoms as shown in Scheme 1.

Inhibitions—Estradiol and estrone inhibit strongly and competitively the dehydrogenation of 5α-androst-1-ene-3,17-dione. The K_i' value of estradiol is 0.25 μM. 3-Hydroxysteroids such as dehydroepiandrosterone and pregn-5-ene-3,20-diol had no effect on the dehydrogenase reaction.

DISCUSSION

In the previous study, we isolated 3-ketosteroid-Δ¹-dehydrogenase from *N. corallina* and characterized the enzymatic and spectrophotometric properties (*1, 2, 4*). It is a typical flavoenzyme catalyzing 1α,2β-axial elimination of hydrogen atoms of 3-ketosteroids and also transhydrogenation between 3-keto-4-ene-steroid and 3-keto-1,4-diene-steroid (*4*). During the work, 3-keto-5α-steroid-Δ⁴-dehydrogenase was also found in crude extract of the organism. A low content of the enzyme, however, made hard the isolation by general chromatographic procedures. We have developed a new affinity gel consisting of 19-nortestosterone coupled to Sepharose 4B and have succeeded in purification of the enzyme. The purified preparation is free from 3-ketosteroid-Δ¹-dehydrogenase and has high Δ⁴-dehydrogenase activity for 5α-androst-1-ene-3,17-dione. Since the reactions of both steroid dehydrogenase are inhibited by estradiol and the inhibition constants (K_i') are very different, $K_i' = 0.25$ μM for the Δ⁴-enzyme and 26.2 μM for the Δ¹-enzyme (*1*), the affinity gel ligand chosen was 19-nortestosterone, which is converted to estradiol by the Δ¹-enzyme. The Δ⁴-dehydrogenase could be eluted effectively from the gel with a steroid-containing buffer.

TABLE III. **Molecular and catalytic properties of 3-ketosteroid-Δ¹-dehydrogenase and 3-ketosteroid-Δ⁴-dehydrogenase.**

Enzyme	Δ¹-Dehydrogenase	Δ⁴-Dehydrogenase
Molecular weight	60,500 (monomer)	60,000 (monomer)
Isoelectric point	about 3.1	4.3
Flavin (per mol)	FAD, 1 mol	FAD, 1 mol
Substrate	Androst-4-ene-3,17-dione	5α-Androst-1-ene-3,17-dione
Product	Androsta-1,4-diene-3,17-dione	Androsta-1,4-diene-3,17-dione
Hydrogen acceptor	Redox dye (PMS) menadione, O_2	Redox dye (PMS) (low reactivity with O_2)
Molecular activity	1.8×10^5 min^{-1}	2.7×10^3 min^{-1}
K_m for 3-ketosteroid	35.7 μM	4.4 μM
pH optimum	10	9-10
Transhydrogenase activity	2,000 min^{-1}	10 min^{-1}

The 3-keto-5α-steroid-Δ⁴-dehydrogenase is a typical flavo-dehydrogenase having 1 mol of FAD per mol of enzyme. Several properties of this enzyme have been studied and are summarized in Table III, which also contains the properties of 3-ketosteroid-Δ¹-dehydrogenase (*1, 2, 4, 5*). There are some similarities and differences between the two enzymes. They are both monomeric molecules with a similar size, but the electrophoretic properties of the native enzymes are quite different, reflecting the difference of their isoelectric points. As regards the enzymatic activity, Δ⁴-dehydrogenase exhibits a lower oxidase activity and a significant amount of cytochrome *c* reductase activity in contrast to the Δ¹-dehydrogenase. Further, a lower transhydrogenase activity is observed with the Δ⁴-enzyme. These differences of the reactivities for electron acceptors may arise from the difference of redox potential of each enzyme-bound FAD molecule. The Δ⁴-enzyme is of a flavo-dehydrogenase type and the Δ¹-enzyme is of a flavo-oxidase type, as discussed in the previous paper (*1, 2*). The natural electron acceptors for the two enzymes are not known but may be flavoprotein-like electron transfer flavoproteins for several dehydrogenase (*14-20*).

Like Δ¹-dehydrogenase, the Δ⁴-dehydrogenase shows a wide substrate specificity for C_{19}- and C_{21}-3-ketosteroids but requires 5α-configuration in the substrate. The type of dehydrogenation of the Δ⁴-enzyme is *trans*-axial elimination, as observed with the Δ¹-enzyme (*4*). This fact suggests, on the basis of the scheme proposed for the Δ¹-enzyme (*4*), that the 4β-hydrogen is abstracted as a hydrogen ion to a nucleophilic residue of the active site through a protein-based reaction and the 5α-hydrogen is transferred as a hydride ion to the N(4)-position of the enzyme-flavin. Transhydrogenation experiments (Fig. 6)

indicated that a 6α-hydroxyl group of a substrate affects the reaction negatively and furthermore, 11-hydroxylated steroid is a less favorable substrate than 11-ketosteroid. A similar effect of an 11-hydroxyl group was observed with 3-ketosteroid-Δ¹-dehydrogenase (*1*). It is not clear why the 11-hydroxyl group prevents the elimination of the 4β, 5α-hydrogens by the Δ⁴-enzyme and also of the 1α,2β-hydrogens by the Δ¹-enzyme. The hydroxyl group is located to the rear of the 1α,2β-hydrogens while the 4β,5α-hydrogens are located at symmetric positions with respect to the C3-C10 axis. The results presumably reflect the structure of the substrate binding sites of these enzymes.

Levy and Talalay reported the presence of 3-ketosteroid-Δ⁴-dehydrogenase with 3-ketosteroid-Δ¹-dehydrogenase in bacteria grown in steroid-containing medium (*6*). 4-Pregnene-17α,21-diol-3,20-dione, an enzyme inducer, is a substrate for 3-ketosteroid-Δ¹-dehydrogenase but not for the 3-ketosteroid-Δ⁴-dehydrogenase. The steroid can only become a substrate in the reverse reaction. In the metabolism of this steroid, the substrate for the Δ⁴-enzyme was not formed. Thus, the steroid that induces the enzyme is not known, and it would be interesting to determine whether 4-pregnene-17α,21-diol-3,20-dione itself is the inducer or not. Some possibilities are (i) contamination by 5α-1-pregnene-17β,21-diol-3,20-dione in the steroid used for induction of the Δ¹-enzyme; (ii) formation of the C4-C5 saturated steroid by metabolism and its effect on the induction of the Δ⁴-enzyme; and (iii) adventitious co-induction of the enzyme with the induction of the Δ¹-enzyme by 4-androstene-3,17-dione, which is a key intermediate in the pathway of steroid metabolism. The first case seems unlikely because the steroid preparation was from a commercial source and though it was used without further purification, it should not contain enough C4-C5 saturated steroid to induce the enzyme which exhibits about one-tenth of the activity of the Δ¹-enzyme. The second case is also unlikely. It is difficult to account for the production of C4-C5 saturated steroids by other reductases in the degradation pathway. The third may be the most probable, though we do not have any data to support this, and at present, it is difficult to reach a clear conclusion. Therefore, the physiological meaning of this enzyme remains to be elucidated.

The availability of the purified Δ⁴-dehydrogenase has enabled us to characterize its role in steroid metabolism. The results presented here and those obtained with the 3-ketosteroid-Δ¹-dehydrogenase (*1-5*) show them to be members of a novel class of steroid dehydrogenases. Most steroid dehydrogenases so far isolated from microbial and also mammalian sources are pyridine nucleotide-linked enzymes and abstract one hydrogen atom from a carbon atom having one hydroxyl group, such as 17β-hydroxysteroid dehydrogenase and others. In contrast, the enzymic properties of the Δ¹- and Δ⁴-enzymes are similar in many respects to those of flavoenzymes such as acyl-CoA dehydrogenase (*20-22*), glutaryl-CoA dehydrogenase (*23*), acyl-CoA oxidase (*24*), and lactate oxidase (*25*). The purified Δ⁴-enzyme has also yielded molecular information about these interesting enzymes as discussed in the previous papers (*1, 2, 4, 5*).

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