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SOME OBSERVATIONS ON THE Δ^1-DEHYDROGENATION OF STEROIDS BY *BACILLUS SPHAERICUS*

VLADIMIR STEFANOVIC*, MIKA HAYANO AND RALPH I. DORFMAN

Worcester Foundation for Experimental Biology Shrewsbury, Mass. (U.S.A.)

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SUMMARY

A study of the Δ^1-dehydrogenation of steroids by intact cells and cell-free preparations of *Bacillus sphaericus* (ATCC 7955) has been made. The catalysis comprises of two phases, first a reversible reaction where a rapid exchange of the hydrogens of C-1α and C-2 of the steroid substrate occurs with the hydrogen of the medium, and the second, the essentially irreversible formation of the Δ^1 bond. Examination of these two phases with the use of tritium-labelled steroids and tritium oxide-enriched water has been made. Various electron acceptors have been tested with a cell-free preparation of Δ^1-dehydrogenase and it has been shown that substances with the quinone structure are highly effective.

INTRODUCTION

Some years ago a study was initiated to determine the stereochemical course of steroid 1,2-dehydrogenation by *Bacillus sphaericus*, one of the numerous microorganisms known to effect this transformation[1]. [1α,2-$^3H_2$]5α-Androstane-3,17-dione was prepared for this purpose. It soon became apparent from fermentations with this compound, and with [1α-^{3}H]5α-androstane-3,17-dione, and [1α-^{3}H]4-androstene-3,17-dione, that steroids thus labelled could not be used to assess the point in question**. Following each experiment the analysis of recovered starting material consistently showed a loss of tritium from the molecule. These results indicated the existence of the equilibrium reaction, androstanedione $\rightleftharpoons$ 1-androstenedione, or 4-androstenedione $\rightleftharpoons$ 1, 4-androstadienedione despite the fact that no reduction of the Δ^1-structures could be demonstrated under conditions used to study the forward reaction. An exploration of this phenomenon with the use of whole cell and cell-free preparations of *B. sphaericus* was henceforth made with labelled steroids and with non-labelled compounds in the presence of tritium oxide enriched incubation media. A separation of two phases of the 1,2-dehydrogenation reaction was obtained in the cell-free system, a first reversible

* Present address: Hoffman-La Roche Co., Nutley, N.J. (U.S.A.).

** The question of the stereochemical course of the reaction was resolved *via* another approach, with the use of steroid structures containing substituent groups at carbons 1α, 2α and 2β (see ref. 2). The results clearly demonstrated a diaxial 1α, 2β hydrogen elimination in the enzymic Δ^1-dehydrogenation by *B. sphaericus*.

step, in which a rapid exchange of the hydrogens of C-1α and C-2 of the steroid occurs with the hydrogen of the medium, and the second step, the essentially irreversible formation of the Δ^1-bond. This manuscript presents a report of these findings and an account of other experiments concerned with electron acceptor systems for the dehydrogenation.

METHODS

*Radioactive steroids**

[1α,2-$^3H_2$]5α-Androstane-3,17-dione was prepared by catalytic reduction of 5α-androst-1-ene-3,17-dione with gaseous tritium under neutral conditions. Aqueous alkaline equilibration of the 1,2-labelled structure yielded [1α-^{3}H]5α-androstanedione, with a loss of approx. 40 % of the initial radioactivity. Of the remaining radioactivity, about 90–95 % is located at C-1α. [1α-^{3}H]4-Androstene-3,17-dione was prepared through bromination–dehydrobromination procedures from its 5α-saturated analogue. All compounds used in this study were purified to constant specific activity through reverse isotope dilution, paper and silica gel chromatography and crystallization.

Fermentation

B. sphaericus (ATCC 7055) was grown on a medium containing 0.3 % Yeast Extract "Difco" and 0.5 % N-Z Case (Peptone "Difco"). 50 mg of steroid dissolved in 3 ml ethanol and 1 ml propylene glycol were generally added to 400 ml of a 24-h culture and fermentation carried out aerobically for the desired length of time with constant shaking at 30°.

Initial time runs showed that our preparations were capable of converting 25–75 % of androstanedione (50 mg/400 ml medium) to the Δ^1-product in 55–65 h. For complete transformation, the fermentation was allowed to continue for 72–80 h. With the same concentration of 4-androstene-3,17-dione, 25–75 % conversion was observed in 5–15 h time. Complete conversion of 4-androstenedione was achieved in 20–25 h. Incubations continued to 48 h showed little loss in the quantity of $\Delta^{1,4}$-product formed.

After fermentation, the broth was extracted several times with 200 ml of ethyl acetate and the extracts combined, washed once with 1 % $NaHCO_3$, twice with water, and concentrated *in vacuo*.

Resolution and purification of steroids were carried out through silica gel column chromatography, paper chromatography, or a combination of both methods. Elution of materials from the columns was accomplished with benzene and benzene–ethyl acetate mixtures of increasing polarity. Androstanedione was generally obtained in the 25:1 fraction, 1-androstenedione in 20:1, 4-androstenedione in 15:1 and 1,4-androstadienedione in 10:1. Some overlap was encountered. The paper chromatographic system of ligroin–propylene glycol was employed. Four or five 20 × 45 cm sheets were used for the purification of the extract obtained from each fermentation. After the period of resolution, the papers were dried by hanging in air and areas containing steroids eluted three times with shaking for 10 min with 100-ml portions of chloroform–methanol (2:1). After evaporation *in vacuo* the residue obtained was dissolved in benzene, chromatographed as above on a silica gel column and the crystalline

* Synthetic procedures to be published.

fractions recrystallized to a constant specific activity, and identified by melting points and infrared analysis when necessary.

Fermentations in the presence of ^3H_2O

To 400 ml of a 24-h culture of *B. sphaericus* was added 100 mg of 4-androstenedione and 1 C of ^3H_2O (1 ml). Aluminum foil was immediately placed over the cotton plug in the flask and the edges sealed with adhering tape. Fermentation was continued with shaking at 30° for 8 h. Initial time runs showed that only a trace of 1,2-dehydrogenated product appears under these conditions before 10 h time. About 50% conversion was noted after 25 h, and complete transformation seen in about 35 h.

Incubations with cell-free preparations

24-h cultures of *B. sphaericus* were harvested by centrifugation and the cells washed twice with cold 0.33 M phosphate buffer (pH 7.4). 2.5 g of bacteria were generally obtained from 400 ml of culture. The bacterial cells were disrupted by sonication with the use of a Raytheon 10 kc magnetostrictive oscillator. An examination of 5, 10 and 20 min sonication times indicated that there was no essential difference in the dehydrogenating activity of the resultant preparations. For this treatment, the cells were resuspended in the buffer at a concentration of 100 mg wet wt./ml. A 10-min exposure period was arbitrarily selected for use throughout the study. The solution was then centrifuged at speeds indicated in the text for 30 min and the supernatant fluid used for incubations. The Spinco Ultracentrifuge was used for forces between 3500 and 105000 × *g* and the Servall Superspeed Angle Centrifuge SS-1 for routine preparations at 7500 × *g*. Protein contents of the fractions were determined with the biuret reaction[3].

Large scale incubations were carried out in 500-ml Erlenmeyer flasks. To 50 mg of steroid dissolved in 4 ml of ethanol–propylene glycol (3:1), were added 125 ml of enzyme solution obtained after centrifugation at forces indicated in the text. When employed, menadione, 20 mg in 0.8 ml of ethanol, was added at this time. In experiments with tritiated water, 0.9 C of ^3H_2O (0.2 ml) was added and a cotton plug immediately placed in the neck of the flask, covered over with a thin rubber sheet and held in place with rubber bands. Incubations were generally carried out for 4 h at 30° with shaking. Extraction, purification and identification of steroids was accomplished in the manner already described under experiments with whole cells. Because of the possibility of the carry-over of high radioactivity from the incubation water in these experiments, the crystalline steroids isolated were generally dried under high vacuum in a Fisher drying apparatus before counting.

Small scale incubations were carried out in 25-ml Erlenmeyer flasks. To 1 mg of steroid dissolved in 0.1 ml ethanol–propylene glycol (2:1), was added 1.5 ml of enzyme extract and electron acceptor, and the volume brought to 2.0 ml with 0.033 M phosphate buffer (pH 7.4). Unless otherwise specified, electron acceptors which were not soluble in aqueous media were added as ethanolic solutions (0.05 ml). Incubations were carried out at 30° for 1 h. The solutions were extracted with chloroform–methanol (2:1), and the eventual residue resolved by paper chromatography in the ligroin–propylene glycol system. Spotting techniques included the use of ultraviolet light, the Zimmerman reaction for 17-ketosteroids, and the isonicotinic acid hydrazide reaction for conjugated ketones[3]. Estimations of quantities were made by comparing intensities

of the colors formed against those from known quantities of standard substances run simultaneously. Quantitative experiments with 4-androstenedione were made with the use of ^{14}C-labelled steroid. 20000 counts/min (1 mg) of substrate were used per incubation flask. The material extracted after the incubation was placed on 2-cm paper strips and resolved as above. After location of the steroids by ultraviolet scanning, the areas were eluted five times for 10 min with 3 ml of chloroform–methanol (2:1). The eluates were placed directly in the counting vials and evaporated under nitrogen to dryness before addition of the scintillation fluid. Total radioactive recoveries were in the range of 80 %.

Alkaline equilibration of steroids

25–50 mg of steroid were heated for 2 h in 15 ml of a boiling 1 % solution of KOH in methanol–water (2:1). The solution was then neutralized with 10 % HCl, extracted with benzene, and the combined extracts washed with water, dried, and evaporated to a small volume for chromatography on silica gel as described above. Generally a single refluxing served to remove all the exchangeable tritium from the steroid.

Oxidation of steroids with 2,3-dichloro-5,6-dicyano-p-benzoquinone

2,3-Dichloro-5,6-dicyano-*p*-benzoquinone (Aldrich Chemical Co.) and the steroid (1:1) were refluxed in benzene at about 75° for 26 h. The cooled benzene solution was decanted and washed with 2 % NaOH to remove color. The crude crystalline material resulting from the organic layer was purified by thin-layer chromatography with silica gel and 20 % ethyl acetate in benzene, and recrystallized from acetone.

Radioactivity

The steroids were dissolved in 15 ml of scintillation liquid (155 mg of 2,5-diphenyloxazole and 4.6 g of 1,4-bis-2(5-phenyloxazolyl) benzene in 1 kg of toluene (Matheson, Coleman and Bell, Special Purity for Scintillation)) and counted in a Packard Tri-Carb liquid scintillation counter Model 314.

RESULTS

Experiments with [1α,2-^{3}H$_2$]5α-*androstane-3,17-dione* (1α, 55 %; 2, 40 %), [1α-^{3}H]-*androstanedione and* [1α-^{3}H]*4-androstenedione.*

An attempt to determine the stereochemical course of 1,2-dehydrogenation was first considered with the use of [1α,2-^{3}H$_2$]androstanedione. Fermentations with *B. sphaericus* were carried out under conditions designed to turnover about 50 % of this substrate to the Δ^1-product. Analyses of the substrate as well as the product isolated after these fermentations invariably showed a loss of radioactivity from both C-1 and C-2. The next series of experiments were carried out with substrates labelled solely at C-1α. Fermentation of these materials also resulted in loss of tritium from the starting material. Typical data are presented in Table I. Since control experiments with the microorganisms inactivated by autoclave treatment always yielded substrate retaining all the original counts, these results pointed to the existence of an enzymically catalyzed equilibrium of the substrate and product. However, no indication of the reversibility of the reaction could be obtained. When 1,4-androstadienedione was fermented with the microorganism under conditions used for the forward reaction,

TABLE I

FERMENTATION OF TRITIATED STEROIDS WITH *B. sphaericus*

See under *Fermentation* for experimental conditions.

Steroid	*Substrate (counts/min/mg)*	*Recovered substrate (counts/min/mg)*	*Loss of counts (%)*
[1α,2,$^3H_2$]Androstanedione (1α, 55%; 2, 40%)	$811 \cdot 10^5$	$50 \cdot 10^5$	94
[1α,2,$^3H_2$]Androstanedione (1α, 55%; 2, 40%)	$270 \cdot 10^5$	$40 \cdot 10^5$	84
[1α,2,$^3H_2$]Androstanedione (1α, 55%; 2, 40%)	$780 \cdot 10^3$	$354 \cdot 10^3$	55
[1α-^{3}H]Androstanedione	$134 \cdot 10^4$	$18 \cdot 10^4$	87
[1α-^{3}H]4-Androstenedione	$125 \cdot 10^4$	$72 \cdot 10^4$	43

the steroid was recovered unchanged. Only under the special conditions of GOODMAN *et al.*[5], anaerobic fermentation following a 7-h aerobic "contact" period with the substrate, 1,4-androstadienedione, was some 4% of this substance reduced at the 1,2-position.

Experiments with ^3H_2O-enriched medium

Experiments were next designed to measure the extent of the enzymic exchange of the hydrogens of the substrate with those of water. A fermentation was carried out in a closed vessel for 8 h, a period previously determined to yield only a trace of $\Delta^{1,4}$-product. The 4-androstenedione recovered, had a very high specific activity of tritium, 3540 counts/min/μmole of steroid. The theoretical uptake on the basis of two exchangeable hydrogens was calculated to be about 10000 counts/min/μmole. Treatment of this steroid with aqueous alkali revealed that 60% of the counts was located in enolizable positions. Fermentation controls with ^3H_2O and steroid alone or with bacterial cells

TABLE II

INCUBATION OF Δ^1-DEHYDROGENASE WITH STEROIDS IN THE PRESENCE OF ^3H_2O

See under *Incubations with cell-free preparations*, Large scale incubations, for experimental conditions.

Steroid	*Enzyme supernatant fraction*	*Incubation time*	*Recovered substrate (counts/min/mg)*	*Recovered substrate, after alkaline equilibration (counts/min/mg)*
Androstanedione	105000 × *g*	4 h	740	
Androstanedione	7500 × *g*	4 h	17650	2600
4-Androstenedione	105000 × *g*	4 h	200	
4-Androstenedione	7500 × *g*	4 h	10000	3940
4-Androstenedione	7500 × *g*	2 h	6500	
4-Androstenedione	7500 × *g*	20 min	1920	
4-Androstenedione	7500 × *g* (boiled enzyme*)	4 h	26	
17α-Methyl-5α-androstan-17β-ol-3-one	7500 × *g*	4 h	2160	475

* Supernatant fraction heated in boiling water for 30 min.

previously inactivated by autoclave treatment yielded steroid containing no more than about 30 counts/min/μmole.

This exchange reaction was then investigated with cell-free preparations of *B. sphaericus*. It has been shown[6] that sonicated extracts containing Δ^1-dehydrogenase must be supplemented with an electron acceptor such as phenazine methosulfate to complete the catalysis. It was soon discovered that the cell-free preparations were capable of the exchange reaction in the absence of added electron acceptor.

The capacity of supernatant preparations obtained at 7500 and 105 000 $\times$ *g* to catalyze the exchange reaction was next examined (Table II). A larger degree of uptake of radioactivity into the steroids from the incubation medium enriched with ^3H_2O took place with the lower speed extract, 15% being located in a stable position in 5α-androstanedione and 39% in 4-androstenedione. A substrate with restricted enolization possibilities (C-2 and C-4), 17α-methyl-5α-androstan-17β-ol-3-one, was also incubated, isolated and equilibrated with alkali. 78% of the radioactivity incorporated was lost.

From a consideration of the known favored direction of enolization of 3-keto-5α-steroids, which is towards C-2, it is almost certain that the exchangeable tritium in the three substrates cited is at C-2. Rigid experimental proof of this point has been obtained by bromination experiments and will be reported at a later date.

Proof of the location of the tritium introduced into the steroid molecule *via* the enzymic exchange reaction with the medium, was obtained through the following reactions. A sample of [^{3}H]4-androstenedione isolated from an exchange reaction was thoroughly equilibrated with alkali to remove all enolizable radioactivity. It was then oxidized with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone to yield the $\Delta^{1,4}$ analogue. It has been shown that this oxidation proceeds with abstraction of the C-1α hydrogen[7], as has the biological course of the dehydrogenation[2]. Of the stable tritium introduced into the steroid molecule from the medium, about 80% was removed on treatment with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone, thus confirming the fact that the enzymically catalyzed exchange reaction involves this position.

Various attempts were then made to find evidence of the reversal of the dehydrogenation reaction other than that indicated in the enzymic exchange of steroid C-1,2 hydrogens with the tritium of the medium. 1,4-Androstadienedione was incubated anaerobically with DPNH, DPNH and menadione, DPNH and Q_9, dithionite, ascorbic acid, and hydroquinone. No trace of the reduced analogue, 4-androstenedione, could be detected. Finally, in a large-scale experiment with 50 mg of purified 1,4-androstadienedione incubated for 4 h with a 7500 $\times$ *g* preparation in the presence of ^3H_2O, a small quantity of 4-androstenedione (800 μg) was isolated. The radioactivity of this material was 51 000 counts/min/mg, a figure representing 50% of the theoretical quantity calculated for the uptake of 2 hydrogens from the medium. The 1,4-androstadienedione recovered from the incubation had an activity of 19 counts/min/mg.

Fractional centrifugation of cell free preparations

A suspension of *B. sphaericus*, sonicated for 10 min, was subjected to centrifugation under various gravitational forces, and the supernatant extracts tested for enzymic activity in the absence and presence of the added electron acceptor, menadione (Table III). Very little dehydrogenation occurred in even low-speed supernatant preparations in the course of a 2-h incubation period in the absence of the electron

TABLE III

Δ^1-DEHYDROGENASE ACTIVITY IN DIFFERENTIALLY CENTRIFUGED PREPARATIONS

See under *Incubations with cell-free preparations*. Small scale incubations, for experimental conditions. Yields are estimated as described.

Centrifugal force × g	Supernatant protein (mg/ml)	Per cent conversion of			
		Androstanedione		4-Androstenedione	
		Without menadione	With 100 μg menadione	Without menadione	With 100 μg menadione
105 000	0.50	—	—	—	100
50 000	0.65	—	5	—	100
25 000	1.20	—	20	—	100
12 500	3.85	—	40	—	100
7500	5.85	Trace	60	Trace	100
4000	6.80	Trace	70	25	100
1	62.50	—	85	100	100

TABLE IV

COMPOUNDS TESTED AS ELECTRON ACCEPTORS FOR Δ^1-DEHYDROGENATION

See under *Incubations with cell-free preparations*. Small scale incubations, for experimental conditions. Substrate, [4-^{14}C]androstenedione.

	Per cent conversion of substrate
Phenazine methosulfate (4 mg)	100
Menadione (50 μg)	100
Menadione (250 μg)	80
Menadione (500 μg)	50
2,6-Dichlorophenolindophenol (200 μg)	40
Vitamin K_1 (200 μg)	35
Sodium-1,2-naphthoquinone-4-sulfonate (200 μg)	17
Quinone (200 μg)	20
Quinhydrone (200 μg)	65
Q_2 (200 μg)	3
Q_9 (200 μg)	45
Q_{10} (200 μg)	45
"Q" from *B. sphaericus* (400 μg)	35
FMN, FAD, DPN, TPN, yeast and liver extracts (100 μg–1 mg)	0

acceptor. With added menadione, a complete transformation of 4-androstenedione was noted in this same time with all preparations. Androstanedione was a less suitable substrate and was converted less readily. Its transformation decreased proportionately with the decreasing quantity of protein found in the supernatant phase when higher centrifugal forces were used.

Electron acceptors

A survey of the efficacy of various compounds as electron acceptors for Δ^1-dehydrogenase was conducted. Typical results are presented in Table IV. The use of phenazine methosulfate has been described in the work with the Δ^1-dehydrogenase of

Pseudomonas testosteroni[6]. Of interest was the efficiency of menadione, which was active in concentrations as low as 25–50 μg/2 ml of incubation volume. Ten-fold quantities of this substance were somewhat inhibitory. Q_2, Q_9 and Q_{10} were also examined. The difficulty of dispersing these lipids in aqueous media is known. From various experiments using Triton-X, Tween 80, isooctane and other dispersing agents, the highest activity, as reported in Table IV was obtained with the use of propylene glycol–ethanol.

Because of the effectiveness of added Q_9 and Q_{10}, an attempt was made to isolate such substances from the microorganism. The method followed was that of LINN *et al.*[8]. From 280 g (wet wt.) of *B. sphaericus* 3.7 mg of an oily substance were obtained. This material had the characteristic ultraviolet absorption before and after reduction with $NaBH_4$ and a mobility parallel to Q_9 in the paper chromatogram system of PAGE *et al.*[9]. A portion of the substance isolated was tested and found to be capable of catalyzing Δ^1-dehydrogenation.

Steroid substrates

To the list of compounds already examined for Δ^1-dehydrogenation by *B. sphaericus* by STOUDT *et al.*[1] and by HAYANO *et al.*[2] can now be added the following observations. 5α-Androstane-3,17-dione, deoxycorticosterone acetate (product, Δ^1-deoxycorticosterone), epicorticosterone, 19-nor-testosterone (product, estrone), and 19-nor-(10β)-5α-androstane-3,17-dione are readily transformed. On the other hand 5β-androstane-3,17-dione, 19-nor-(10α)-5α-androstane-3,17-dione, and 10-hydroxymethyl-octal-(9)-en-2-one, were recovered unchanged after fermentation.

DISCUSSION

The steroid Δ^1-dehydrogenase from cell-free extracts of *Pseudomonas testosteroni*[6] and *Nocardia restrictus*[10] have been studied. To these can now be added the present investigation with *B. sphaericus*. Indications of the reversibility of the 1,2-dehydrogenation reaction were noted early in our work with both cellular and cell-free preparations of this microorganism. In experiments with (a) tritium-labeled substrates (b) tritium oxide enriched media, a ready exchange of radioactivity between the substrate and media was observed. Further study indicated that the dehydrogenase catalysis was divided into two parts, the first, in which a rapid and reversible exchange of the C-1α and C-2 hydrogens of the steroid substrate occurred with the hydrogen of the medium, and the second part, the essentially irreversible removal of the steroid hydrogens to form the Δ^1 bond. The latter phase could be minimized in whole cell fermentations by limiting aeration, and the two steps separated in cell-free extracts through sonic disruption of cytoplasmic particulates. The protein component(s) necessary for the formation of the Δ^1 product was not sedimented at 105 000 $\times$ *g*, as this supernatant was fully capable of the dehydrogenation in the presence of added electron acceptor. Particulates sedimented at this force contained components which catalyzed the substrate–media hydrogen exchange. Proof of the involvement of the C-1α hydrogen of the steroid substrate was obtained through chemical reactions of known stereospecificity. That the point of equilibrium of the second step of the reaction, the formation of the Δ^1 bond, is far to the right is seen in the lack of observable quantities of reduced product after incubations of Δ^1 steroid substrates. To establish

the reversibility of this step, it was necessary to carry out a large scale incubation with pure 1,4-androstadienedione in the presence of ^3H_2O. [^{3}H]4-Androstenedione with very high specific activity was isolated in a yield of about 1.5 %.

It is evident that the Δ^1-oxidase system consists of the Δ^1-dehydrogenase and an electron transport system. The individual member(s) of the transport system have not been completely elucidated. It was interesting to note the efficiency of the quinone type structures as electron acceptors and for this reason an attempt was made to isolate natural quinones from *B. sphaericus*. A small amount of material with characteristics of Q_9 was found. Whether this substance is an actual member of the Δ^1-dehydrogenase electron acceptor system cannot be established conclusively without further study. The exchange of substrate hydrogen with that of the medium has been observed previously and appears to be mediated through flavin coenzymes[11,12]. Evidence is present that a flavin prosthetic group is a part of the Δ^1-dehydrogenase system[6,10].

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