FURTHER CHARACTERIZATION OF THE 5α-HYDROXYSTEROL DEHYDRASE OF YEAST[1]

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Under conditions now established for measurement of the rate of dehydration, ergosterol has been shown to be formed at equal rates from 5α,8α-epidioxyergosta-6,22-dien-3β-ol and ergosta-7,22-diene-3β,5α-diol substrates. Furthermore, 3α-^{3}H is lost during formation of ergosterol from the 5α-hydroxysterol substrate. Thus, stepwise loss of the leaving group from the 5α position, followed by stabilization of an intermediate by loss of the α-proton from 3-C, is proposed.

INTRODUCTION

Anaerobic formation of ergosterol from ergosta-7,22-diene-3β,5α-diol was demonstrated with broken cell preparations of yeast (1). The particle-bound enzyme that catalyzes dehydration of 5α-hydroxysterol was isolated in soluble form from yeast, extensively purified, and partially characterized (2).

The present report describes further work on characterization of the dehydrase, which provides basis for proposal of a mechanism for the dehydration reaction.

EXPERIMENTAL PROCEDURES

The procedures for the preparation of unlabeled ergosta-7,22-diene-3β,5α-diol and 5α,8α-epidioxyergosta-6,22-dien-3β-ol have been described (1,2).

3α-^{3}H-Ergosta-7,22-diene-3β,5α-diol was prepared by oxidizing the

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3β-hydroxyl group of unlabeled diol to the ketone with tert-butyl chromate (3). The 3-ketone was reduced to the alcohol with $LiAl^3H_4$. The resulting 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol was acetylated and purified as described for unlabeled ester (1,2). The 3α-^{3}H-3β-acetoxyergosta-7,22-dien-5α-ol (4.62×10^5 dpm/mg) thus obtained was homogeneous. ^{3}H-Ergosterol acetate was absent. Thin-layer chromatography on silica gel plates revealed a single, radiochemically-pure compound.

A cell-free extract of baker's yeast (S. cerevisiae, from Standard Brands, Inc., New York) was prepared, and the 5α-hydroxysterol dehydrase was purified as described earlier. Three methods of assay for dehydrase activity employed in this study have been described (2). Substrates were prepared either as suspensions in 0.05 M Tris-HCl buffer (pH 7.4 and containing 10 mM 2-mercaptoethanol) with the aid of Tween 80, or as solutions in propylene glycol (1,2). Enzymic assays were carried out anaerobically at 37°, pH 7.4. All of the procedures summarized above have been described in detail (1,2).

RESULTS AND DISCUSSION

In a direct spectrophotometric assay, enzyme of the highest purity, obtained from extraction of calcium phosphate gel, exhibited constant initial velocities for steady-state kinetic analyses when ergosterol formation was measured at 282 nm; in addition, velocity and enzyme concentration were proportional (2). Thus, in subsequent work a double reciprocal plot of velocity and substrate (ergosta-7,22-diene-3β,5α-diol) concentration yielded a linear relationship for incubation of 10 minutes duration (Fig. 1A). The kinetic parameters calculated from this plot were: K_m, 1.7×10^{-4} M; V_{max}, 77×10^{-9} moles/10 min/mg of protein. Similar experiments were carried out with enzyme carried to an intermediate stage of purity (following Sephadex G-100 gel filtration); ergosterol formation was assayed following extraction of ergosterol into solvent. A linear relationship also was observed in this double reciprocal plot (Fig. 1B). The value for K_m, calculated from this plot, was also 1.7×10^{-4} M. These results provide the steady-state kinetic parameters for the 5α-hydroxy-

sterol dehydrase and verify the reliability of both spectrophotometric methods of assay.

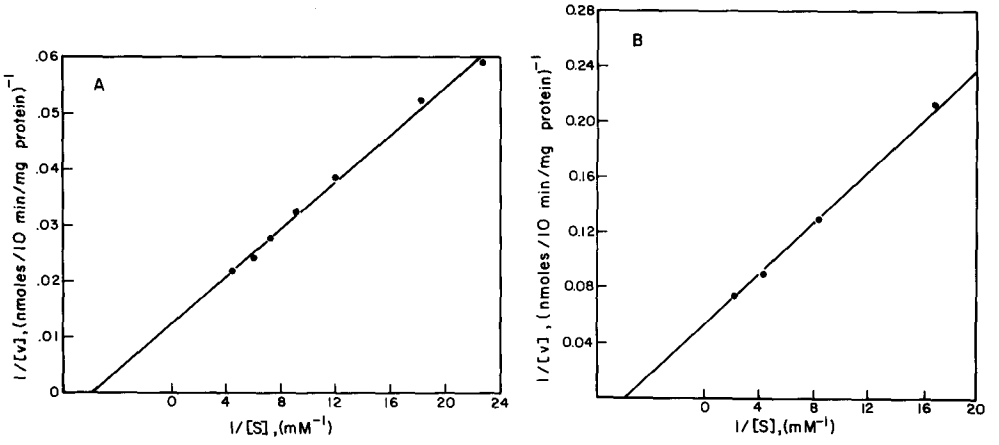

Figure 1. Lineweaver-Burk Plots for Dehydration. A. Each cuvette contained 1 ml (0.17 mg protein) of the dehydrase preparation. Each point represents the average of the results of duplicate assays. B. Each incubation flask contained 5.04 mg protein. The final incubation medium was 60% propylene glycol, and the final incubation volume was 5.2 ml. Incubations were carried out at 37° for 10 min. Each point represents the average of the results from duplicate sets of incubations.

Hamilton and Castrejon (4) earlier suggested that 5α,8α-epidioxyergosta-6,22-dien-3β-ol might be a precursor of the $\Delta^{5,7}$-diene system of ergosterol in yeast sterol biosynthesis. Thus, 5α,8α-epidioxyergosta-6,22-dien-3β-ol was tested as a possible substrate for the 5α-hydroxysterol dehydrase. The amounts of ergosterol formed anaerobically from the sterol peroxide and the 5α-hydroxysterol were compared with the various preparations of enzyme obtained at several stages of purification, and various assays were used. Ergosterol formation was equivalent with either 5α,8α-epidioxyergosta-6,22-dien-3β-ol or ergosta-7,22-diene-3β,5α-diol at all stages of enzyme purification examined (Table I). Addition of cofactors was not required with either substrate. These results suggest that the same enzyme may catalyze the formation of ergosterol from both oxysterols and that the oxygen function at 5-C may not have to be a hydroxyl group.

In our earlier report (2), the stoichiometry of the enzymic dehydration

TABLE I

Conversion of 5α,8α-Epidioxyergosta-6,22-dien-3β-ol and Ergosta-7,22-diene-3β,5α-diol into Ergosterol*

Enzyme preparation	Substrate	
	5α,8α-Epidioxy-ergosta-6,22-dien-3β-ol	Ergosta-7,22-diene-3β,5α-diol
	nmoles ergosterol formed/mg protein	
Microsomes	8.1	7.5
Enzyme preparation following Sephadex G-100 gel filtration	31.7	33.2
Enzyme preparation following calcium phosphate gel treatment	520.0	483.0

*Each flask contained, in 3.2 ml, enzyme, 500 nmoles of substrate, 4 mg of glucose oxidase, and 60 mg of glucose. Incubations were for 40 min. Enzymic assays were carried out by the indirect spectrophotometric method (2). Values represent results of duplicate flasks in two separate incubations each.

reaction was measured by two independent methods. With both methods, the rates of disappearance of substrate and formation of product were equal. Stoichiometric measurements were then carried out with the more easily synthesized 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol (rather than ^{14}C-labeled) as substrate, and enzyme preparations at two widely different states of purification were used to further substantiate the stoichiometry. There was an unexpected, striking loss of tritium from 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol (Table II) when a crude preparation of enzyme was used. Furthermore, no tritium radioactivity was recovered in the product, ergosterol. Indeed, the amount of substrate disappearance, calculated from tritium loss, was equivalent to the amount of ergosterol formed. Thus, to eliminate possibility of side reactions causing tritium loss, in experiment II partially-purified enzyme was used, and ergosterol formation was measured simultaneously with radiochemical analysis of tritium loss. The amount of ergosterol formed was approximately equal to the amount of ergosta-7,22-diene-3β,5α-diol lost. These results thus indicated that 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol was converted enzymically to ergosterol, and complete

TABLE II

Incubation of 3α-^{3}H-Ergosta-7,22-diene-3β,5α-diol*

Experiment #	Disappearance of 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol		Formation of ^{3}H-ergosterol		Spectrophotometric assay of ergosterol formation†
	dpm lost	nmoles converted	dpm incorporated	nmoles formed	nmoles formed
I	33,384	160	141	0.65	---§
II	28,617	155	0	0	145

*Radiochemical assays were performed with 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol as previously described for ^{14}C-ergosta-7,22-diene-3β,5α-diol (2). All values were corrected for recovery and are averages of the results of duplicate flasks. Incubations were carried out at 37° for 40 min. Experiment I: Each flask contained 0.2 mg of 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol added as a Tween 80 suspension in Tris buffer. Each flask also contained 39.0 mg protein of the crude homogenate, 60 mg of glucose, and 4 mg of glucose oxidase. The final incubation volume was 3.2 ml. Experiment II: Each flask contained 1 mg of 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol added as a solution of propylene glycol. Each flask contained 4.56 mg protein of the partially-purified enzyme preparation, 4 mg of glucose oxidase, and 60 mg of glucose. The final incubation volume was 5.2 ml, and the final incubation medium was 60% in propylene glycol.

†The same enzyme preparation incubated with 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol and assayed for ergosterol formation with the indirect spectrophotometric method.

§No spectrophotometric assay was performed with the crude homogenate.

loss of 3α-^{3}H from substrate may have accompanied the dehydration.

The results with 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol, and the earlier stoichiometric measurements with ^{14}C-ergosta-7,22-diene-3β,5α-diol (2) are consistent with the mechanism proposed in Figure 2 (I→IV). This mechanism, which includes the formation of a cyclopropane ring between 3-C and 5-C, accounts for the equivalent rate of ergosterol formation and loss of tritium from 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol. Chemical precedents for the formation of a cyclopropane ring within ring A of sterols have been established (5,6). Furthermore, cis-elimination of the elements of water from 5-C and 6-C, which we proposed earlier, not only appears to be less attractive than the two-step 3-C, 5-C dehydration and rearrangement shown in Figure 2, but loss of 3α-^{3}H was not known at the time of that proposal.

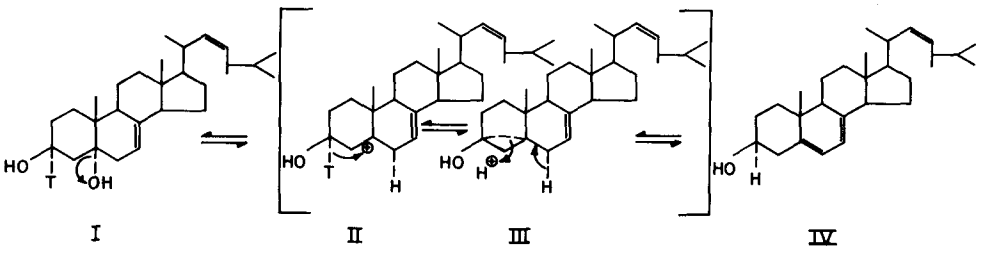

Figure 2. Possible Reaction Mechanism for the Conversion of Ergosta-7,22-diene-3β,5α-diol to Ergosterol.

Evidence for a 5-C oxygenated biosynthetic precursor of ergosterol has not been forthcoming, although the requirements for oxygen (7) and pyridine nucleotide (8) in these terminal reactions is consistent with the possibility of mixed-function oxidation at 5-C. Existence of the dehydrase should not be construed as evidence for or against such a pathway. However, this further information on the dehydrase and the important observation of tritium loss explains an existing paradox. Akhtar and Parvez (9) added 3α-^{3}H-ergosta-7,22-diene-3β, 5α-diol to anaerobically-growing yeast cells, and no ^{3}H-radioactivity was incorporated into ergosterol. Thus, lack of incorporation of 3α-^{3}H into ergosterol was interpreted by the workers as evidence against a hydroxylation-dehydration pathway in the formation of the $\Delta^{5,7}$-diene system of ergosterol. If the dehydrase catalyzes intracellular formation of ergosterol from 3α-^{3}H-ergosta-7,22-diene-3β,5α-diol, retention of ^{3}H would not be expected (Table II). Furthermore, other 5-oxygenated substrates may be converted into ergosterol (Table I) when incubations are carried out with comparable concentrations of substrate (Fig. 1). Thus, we feel that data presented to date neither unequivocally demonstrate nor deny obligatory formation of 5-oxygenated intermediates of ergosterol biosynthesis.

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