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LOCALIZATION OF 3β AND 17β-HYDROXYSTEROID DEHYDROGENASE IN *PSEUDOMONAS TESTOSTERONI*

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SUMMARY

Subcellular distribution studies of 3β and 17β-hydroxysteroid dehydrogenase activity in *Pseudomonas testosteroni* revealed that much of the enzyme released from whole cells is in a soluble state. Some of the 3β and 17β-hydroxysteroid dehydrogenase activity does, however, remain bound to membrane vesicles. The enzyme is present *in vivo* either in both membrane-bound and soluble states or the enzyme is loosely membrane-bound and becomes solubilized during subfractionation. The 1-ene-dehydrogenase, on the other hand, appears to be more firmly membrane-bound. Antibody to the 3β and 17β-hydroxysteroid dehydrogenase inhibited 3β and 17β-hydroxysteroid dehydrogenase to the same extent in intact or disrupted spheroplasts, indicating that the enzyme was equally accessible in both cases. The 3β and 17β-hydroxysteroid dehydrogenase is probably loosely bound to the outer surface of the cytoplasmic membrane.

INTRODUCTION

We have shown that the 3β and 17β-hydroxysteroid dehydrogenase present in membrane vesicles prepared from *Pseudomonas testosteroni* is involved in the active uptake of steroids by the membrane vesicles [1, 2, 3]. Recent studies with an antibody of the purified 3β and 17β-hydroxysteroid dehydrogenase indicated that the dehydrogenase in membrane vesicles was accessible to the antibody [4]. We concluded that the enzyme was probably localized on the outer surface of the membrane vesicles. One of the criticisms levied against the use of membrane vesicles for study of transport of solutes across membranes is that the orientation of the vesicle may not be the same as in the intact organism [5]. In this paper we present two types of experimental evidence which suggest that the 3β and 17β-hydroxysteroid dehydrogenase is localized on the outer surface of the cytoplasmic membrane of *Ps. testosteroni.* The first is inhibition of the spheroplast enzyme activity by anti-3β and 17β-hydroxysteroid dehydrogenase and the second is the subcellular distribution of the enzyme.

The presence of a 1-ene-dehydrogenase activity in membrane vesicles from *Ps. testosteroni* has been implied because androstenedione* generated during transport of testosterone or dehydroepiandrosterone is transformed to androstadienedione [2]. We have studied the distribution of this enzyme in *Ps. testosteroni* and have found that it is more tightly membrane-bound than the 3β and 17β-hydroxysteroid dehydrogenase.

* Trivial and systematic nomenclature of steroids: Androstenedione = 4-androstene-3,17-dione; Androstadienedione = 1,4-androstadiene-3,17-dione; Dehydroepiandrosterone = 3β-hydroxy-5-androsten-17-one; Testosterone = 17β-hydroxy-4-androsten-3-one.

EXPERIMENTAL PROCEDURES

Materials

Ps. testosteroni 11996 was obtained from American Type Culture Collection, Rockville, Maryland; unlabelled testosterone from Steroloids, Pawling, New York; NAD^+ from Schwarz-Mann, Orangeburg, New York; [1, 2-^{3}H]-testosterone from New England Nuclear Corporation, Boston, Massachusetts; Vestapol W from Polysciences, Inc., Warrington, Pennsylvania. The anti-3β and 17β-hydroxysteroid dehydrogenase [7] was generously supplied by Dr. Richard Schultz, Harvard Medical School.

Methods

Media used for growth and conditions for induction of binding activity have been described [1].

Preparation of subcellular fractions. Membrane vesicles were prepared after spheroplast formation using the lysozyme-EDTA method, as described by Kaback[6]. Cells were harvested by centrifugation at 12,000 *g* for 10 min. The pellet was washed by resuspension in 10 mM Tris–HCl, pH 8.0, at 0°C. After centrifugation at 12,000 *g* for 10 min, the cells were resuspended in 30 mM Tris–HCl, pH 8.0, containing 20% sucrose (80 ml/g cells). The suspension was stirred for 30 min. at room temperature, and then disodium ethylenediaminetetraacetate (2Na·EDTA) and lysozyme were added to final concentrations of 10 mM and 0.5 mg/ml respectively. The suspension was stirred for a further 30 min. at room temperature and centrifuged at 23,000 *g* for 20 min. This treatment

releases the constituents of the periplasmic space, the area between the two membranes which make up the cell envelope in gram negative bacteria. Thus the supernatant is the Periplasmic Fluid and the pellet is the Spheroplast fraction.

Using approximately 0.50 ml/g wet wt. bacteria, 0.1 M potassium phosphate buffer, pH 6.6, containing 20% sucrose and 20 mM $MgSO_4$, the spheroplast fraction was resuspended after adding RNAse and DNAse to final concentrations of 3 mg/ml. The suspension was then poured into 200–500 times its volume of 50 mM potassium phosphate buffer, pH 6.6, at 37°C and this suspension was incubated at 37°C for 15 min. with vigorous stirring before adding 2Na·EDTA, pH 7.0, to a final concentration of 10 mM. After further stirring for 15 min. at 37°C, $MgSO_4$ was added to a final concentration of 15 mM and the suspension was stirred for 15 min. more at 37°C. The suspension was then centrifuged at 23,500 *g* for 20 min. The supernatant is the Osmotic Shock Fluid.

The pellet was homogenized in 0.1 M potassium phosphate buffer, pH 6.6, containing 10 mM 2Na·EDTA at 0°C, and the suspension was centrifuged at 4000 *g* for 30 min. The supernatant was decanted and recentrifuged at 4000 *g* for 30 min. The pellets from these two centrifugations were combined and are called Cell Debris.

The supernatant was subjected to a centrifugation at 60,000 *g* for 30 min. The pellet is the Membrane Vesicle fraction which was resuspended in 0.1 M potassium phosphate, pH 6.6 and stored in liquid nitrogen. The supernatant is the Membrane Vesicle Supernatant.

Disruption of cell fractions was done in a French pressure cell at 17,000 $lb/inch^2$ or by sonication at 21 KHz for two 20-s intervals in conical tubes which were kept cold.

Antibody studies. Spheroplasts were prepared as described above and resuspended in 50 mM potassium phosphate, pH 6.6, containing 0.29 M CsCl. The spheroplasts (25 μg protein) were incubated at room temperature for 30 min. and then overnight at 2°C in 0.60 ml containing 25 mM potassium phosphate with increasing amounts of antibody [7] in the presence or absence of 0.29 M CsCl. The contents of each tube were then assayed for 3β and 17β-hydroxysteroid dehydrogenase. Those spheroplasts which had been incubated with CsCl were assayed in the presence of 0.29 M CsCl.

Electron microscopy. Spheroplasts which had been incubated overnight in the presence and absence of CsCl, as described above, were prepared for electron microscopy by fixation of the spheroplast pellet (23,500 *g* for 20 min.) in glutaraldehyde, followed by OsO_4 [8] and embedded in Vestapol W. Thin sections were stained with uranyl acetate and lead citrate [8].

Enzyme assays. The assay for 3β and 17β-hydroxysteroid dehydrogenase was performed as described by Talalay[9]. One unit of enzyme was defined as reduction of 0.48 nmol NAD^+ under the specified conditions.

The 1-ene-dehydrogenase activity was determined by measuring the conversion of [1,2-^{3}H]-testosterone to ^3H_2O by a method previously described [10].

Protein was determined by the method of Lowry *et al.*[11].

Polyacrylamide gel electrophoresis was performed as previously described [7].

RESULTS

Subcellular distribution of 3β and 17β-hydroxysteroid dehydrogenase

Results from previous experiments indicated an association of a 3β and 17β-hydroxysteroid dehydrogenase activity with membrane vesicles derived from *Ps. testosteroni* [1–3]. That the previously purified and characterized enzyme is indeed physically associated with the membrane vesicles was shown by preparing membrane vesicles and sonicating them to liberate the enzyme. The solution was then centrifuged for 2 h at 100,000 *g* to remove membrane fragments. The supernatant which contains essentially all of the enzymatic activity was subjected to polyacrylamide gel electrophoresis. Figure 1 shows that the four activities with the lowest mobilities in the polyacrylamide gel (bands 1–4) which correspond to the characterized 3β and 17β-hydroxysteroid dehydrogenase [7] were present in the membrane vesicle preparation. It is interesting to note that two other steroid dehydrogenases that are present in crude extracts of *Ps. testosteroni* induced with testosterone are also associated with the membrane vesicles (bands 5 and 6) [7].

Though some of the 3β and 17β-hydroxysteroid dehydrogenase is associated with the membrane fraction, the quantitative subcellular distribution of the enzyme was not known. The distribution of the enzyme was determined by collecting subcellular fractions generated during the preparation of the membrane vesicles and assaying for the 3β and 17β-hydroxysteroid dehydrogenase. In the initial studies, two different methods for disrupting the cells were employed to ensure that all of the 3β and 17β-hydroxysteroid dehydrogenase activity was being measured. As is evident in Table 1, the enzyme activity is underestimated when intact bacteria are assayed. Total enzyme activity is increased with spheroplast formation or following disruption of cells with sonication or French pressing. Sonication increased the activity 1.2-fold, while French pressing increased the activity 14.8-fold. When a similar analysis was done on the spheroplast and cell debris fractions (Table 1), it was observed that sonication caused a slight decrease in the enzyme activity of these two fractions. French pressing, on the other hand, caused an increase in enzyme activity of the spheroplasts (3.6-fold) and of the cell debris fraction (3.8-fold). The increase was

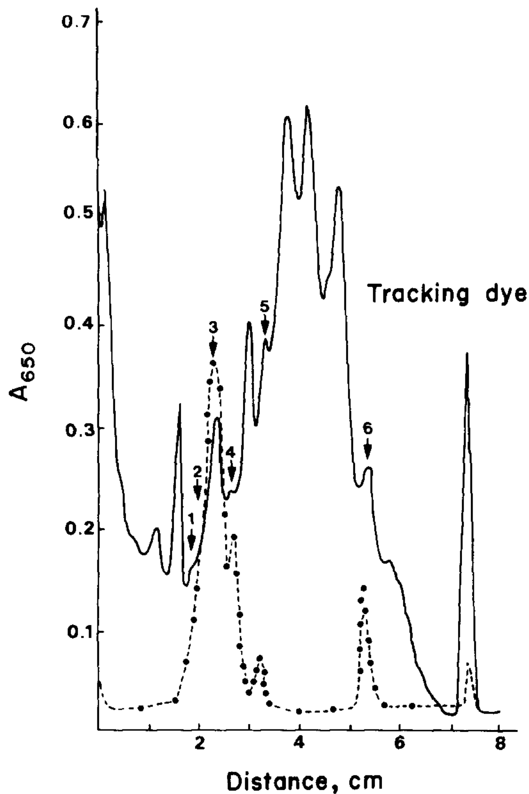

Fig. 1. Gel electrophoresis of membrane proteins. The vesicles were prepared according to the method of Kaback[6], frozen in dry-ice/acetone and stored at −70°C. The vesicles were thawed and sonicated and the solution centrifuged at 100,000 g for 2 h. The supernatant, 60 μl of A_{280} 2.92, was subjected to electrophoresis in a 5% polyacrylamide gel, pH 8.9, and stained for protein (——) or for enzyme activity (●---●) [7]. Bands 1–4 correspond to 3β and 17β-hydroxysteroid dehydrogenase, bands five and six are two other 17β-steroid dehydrogenases present in both the bacterium and membrane preparation. These bands were clearly resolved on the gels.

less than that observed with whole cells. Therefore, it was decided to French press these three fractions before assaying for 3β and 17β-hydroxysteroid dehydrogenase activity. A second pass through the French press did not increase the activity in these fractions. The activity of other fractions subjected to French pressing was not increased.

Table 2 shows the subcellular distribution of the 3β and 17β-hydroxysteroid dehydrogenase in the various fractions. Eighty-one % of the whole cell 3β and 17β-hydroxysteroid dehydrogenase was recovered. Most of the activity was found in the periplasmic fluid (34%) and in the cell debris fraction (38%). The cell debris fraction consists mainly of unbroken cells and re-treatment of this fraction with lysozyme–EDTA may lead to a higher yield of membrane vesicles. Only 5.5% and 0.6% of the enzyme was found in the membrane vesicle supernatant fraction and in the membrane vesicle fraction, respectively. Examination of the relative specific activities shows that the membrane vesicle supernatant fraction and the periplasmic fluid fraction are the fractions enriched in 3β and 17β-hydroxysteroid dehydrogenase relative to the whole cell fraction. The membrane vesicle supernatant fraction is enriched 6.1-fold while the periplasmic fluid is enriched 1.3-fold.

The results of this subcellular distribution indicate that the 3β and 17β-hydroxysteroid dehydrogenase is either localized both in the periplasmic space and bound to cytoplasmic membrane, or that it is loosely bound to cytoplasmic membrane and is released into the periplasmic space during formation of spheroplasts. Final centrifugation leading to harvesting of membrane vesicles or subsequent centrifugations of vesicle preparations result in solubilization of the 3β and 17β-hydroxysteroid dehydrogenase. This leads us to conclude that the membrane-bound fraction of the 3β and 17β-hydroxysteroid dehydrogenase is loosely bound, and is solubilized by centrifugation in the presence of EDTA.

Membrane vesicles prepared from induced cultures appear to have associated 1-ene-dehydrogenase activity [2]. It was therefore of interest to determine its subcellular distribution and orientation on the membrane. Unlike that of 3β and 17β-hydroxysteroid dehydrogenase, it was possible to recover only about 10% of the 1-ene-dehydrogenase activity present in intact bacteria. Periplasmic fluid contained only 0.1% of the activity, membrane vesicle supernatant 0.003% and membrane vesicles 0.002%. However, Table 3 shows that, in three experiments, the relative specific activity of the 1-ene-dehydrogenase was 6 to 13-fold higher in the membrane vesicle fraction than in the

Table 1. Effect of sonication and French pressing on the activity of the 3β and 17β-hydroxysteroid dehydrogenase activity of whole cells, spheroplasts and cell debris

	3β and 17β-hydroxysteroid dehydrogenase activity (nmol NADH/min/mg protein)		
	Without treatment	Sonication	French pressing
Whole cells	8.0	9.6	118.2
Spheroplasts	33.5	23.2	83.4
Cell debris	12.4	10.4	39.4

Note: 3β and 17β-hydroxysteroid dehydrogenase was assayed as described in Methods. Sonication and French pressing were performed as described in Methods. The results are the average of two fractionations.

Table 2. Subcellular distribution of 3β and 17β-hydroxysteroid dehydrogenase

	Protein		3β and 17β-Hydroxysteroid dehydrogenase		
	(mg)	(%)	Units NADH produced/min $\times 10^{-3}$	(%)	Relative specific activity
Whole cells	665	100	215.04	100	1.00
Periplasmic fluid	170	25.6	73.11	34.0	1.33
Osmotic shock fluid	151	22.7	5.81	2.7	0.12
Cell debris	311	46.7	81.29	37.8	0.81
Membrane vesicle supernatant	6	0.9	11.83	5.5	6.10
Membrane vesicle	14	2.1	1.29	0.6	0.28
Recovery	·	98.0		80.6	

Note: One litre of cells was grown for this study. The fractionation procedure and assay for 3β and 17β-hydroxysteroid dehydrogenase were performed as described in Methods. The whole cells and cell debris were French pressed before being assayed. Relative specific activity is based on the whole cell fraction, after French pressing, as unity.

membrane vesicle supernatant fraction. The relative specific activity of the 3β and 17β-hydroxysteroid dehydrogenase, on the other hand, was 22 to 59-fold higher in the membrane vesicle supernatant fraction than in the membrane vesicle fraction. The 1-ene-dehydrogenase appears to be more tightly bound to the membrane, under conditions of these preparations, than 3β and 17β-hydroxysteroid dehydrogenase.

Inhibition of spheroplast 3β and 17β-hydroxysteroid dehydrogenase

In an attempt to correlate the localization and orientation of 3β and 17β-hydroxysteroid dehydrogenase in membrane vesicles and in the intact organism, we studied the effect of anti-3β and 17β-hydroxysteroid dehydrogenase on the enzyme activity. We used spheroplasts prepared by the lysozyme–EDTA method rather than whole cells, as spheroplasts prepared in this way have large areas of unmasked inner membrane [12]. Therefore, if the enzyme were localized on the outer surface of this membrane it would be accessible to the antibody. Others [13] have used accessibility of antibody to enzymes in spheroplasts as an indication of localization of enzymes.

For this type of localization study, it is important to ensure that spheroplasts retain their integrity during the incubation with the antibody. Figure 2 shows that many intact spheroplasts could be observed by electron microscopy after incubation of the spheroplasts in 0.29 M CsCl overnight (A) compared to those spheroplasts incubated in the absence of CsCl (B). We also attempted to protect spheroplasts from disruption with iso-osmolar solutions of sucrose or glycerol but found that more lysis of spheroplasts occurred in these solutions than in CsCl. Futai and Tanaka[13] also found that keeping spheroplasts intact in sucrose was difficult.

When spheroplasts either in the presence or absence of CsCl were incubated with anti-3β and 17β-hydroxysteroid dehydrogenase considerable inhibition (70%) of the 3β and 17β-hydroxysteroid dehydrogenase could be achieved (Fig. 3). There was no significant difference in the effect of the antiserum on the intact or disrupted spheroplasts.

DISCUSSION

The membrane-bound 3β and 17β-hydroxysteroid dehydrogenase is intimately involved in the uptake of steroids by membrane vesicles of *Ps. testosteroni*. In order to understand all of the events occurring during the uptake process in membrane vesicles, it is important to know the orientation of the com-

Table 3. Relative specific activities of 3β and 17β-hydroxysteroid dehydrogenase and 1-ene-dehydrogenase in the membrane vesicle supernatant fraction and in the membrane vesicle fraction

	Relative specific activity			
	3β and 17β-hydroxysteroid dehydrogenase		1-ene-dehydrogenase	
	M.V.S.	M.V.	M.V.S.	M.V.
Experiment 1	6.13	0.28	0.11	0.67
Experiment 2	4.75	0.17	0.07	0.90
Experiment 3	4.74	0.08	0.01	0.07

Note 1. 3β and 17β-hydroxysteroid dehydrogenase and 1-ene-dehydrogenase were assayed as described in Methods.

Note 2. Relative specific activity is based on whole cell fraction, after French pressing, as unity.

Note 3. M.V.S., membrane vesicle supernatant; M.V., membrane vesicle.

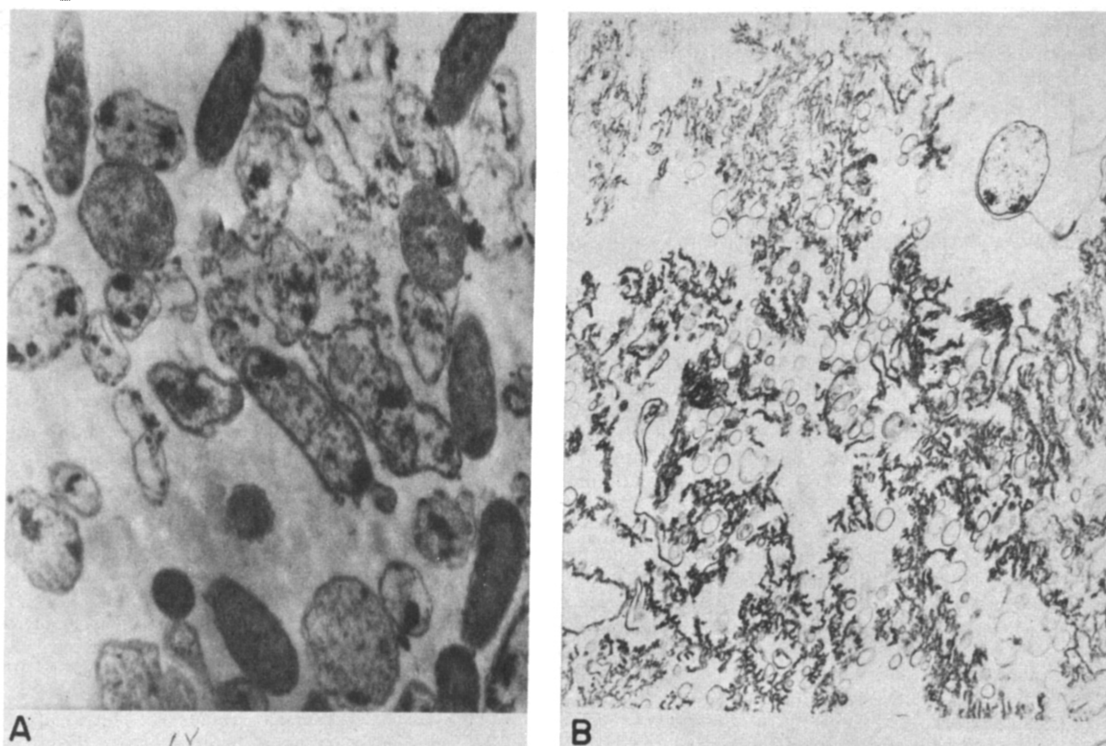

Fig. 2. Electron micrographs of spheroplasts after incubation at room temperature for 30 min. and then overnight at 2°C. (A) in the presence of 0.29 M CsCl. (B) in the absence of CsCl.

ponents involved and to relate these to their orientation in the intact organism. Previous studies had demonstrated the accessibility of the 3β and 17β-hydroxysteroid dehydrogenase to the antibody in the membrane vesicle. This indicated that the enzyme was outward facing in the membrane vesicle. Studies reported here show that the enzyme was equally accessible to the antibody in intact or disrupted spheroplasts indicating that the enzyme is probably located on the external surface of the cytoplasmic membrane facing the periplasmic space. These studies also indicated that the orientation of the membrane vesicles was similar to that of the intact organism. Not all of the enzyme activity, however, could be inhibited by the antibody. Formation of spheroplasts by lysozyme·EDTA treatment probably does not succeed in unmasking all of the cytoplasmic membrane.

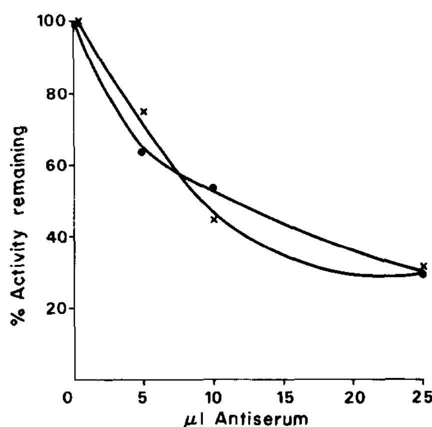

Fig. 3. Effect of increasing amounts of anti-3β and 17β-hydroxysteroid dehydrogenase on spheroplast 3β and 17β-hydroxysteroid dehydrogenase activity. Spheroplasts were prepared as described in Methods and resuspended in the presence (× —— ×) or absence (●——●) of 0.29 M CsCl. Those which had been suspended in CsCl were assayed for 3β and 17β-hydroxysteroid dehydrogenase in the presence of 0.29 M CsCl.

Subcellular distribution studies indicated that the enzyme is probably loosely bound to the outer surface of the plasma membrane. Whether a portion of the enzyme is truly located in the periplasmic fluid or whether that localization occurs as a result of the preparation procedures has not been investigated. In contrast, it appears that the 1-ene-dehydrogenase, also present in membrane vesicles and involved in steroid metabolism [2] is more firmly bound to the membrane.

The binding of steroid to the 3β and 17β-hydroxysteroid dehydrogenase and subsequent dehydrogenation of the steroid at the outer surface of the cytoplasmic membrane may well be early steps in steroid transport across the plasma membrane. The involvement of the electron transport chain [16] may be oxidation of the NADH produced as a result of the activity of the 3β and 17β-hydroxysteroid dehydrogenase.

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