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EXTRACTION OF A STEROID TRANSPORT SYSTEM FROM *PSEUDOMONAS TESTOSTERONI* MEMBRANES AND INCORPORATION INTO SYNTHETIC LIPOSOMES

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Summary—A steroid binding protein has been extracted from *Pseudomonas testosteroni* membranes with an organic solvent system. This protection binds some C19 and C21 steroids but not C18 steroids. When this protein is incorporated into synthetic lipid vesicles constructed from *P. testosteroni* phospholipids, the vesicles perform concentrative uptake of testosterone in the presence of the ionophore valinomycin. This steroid binding protein is thus believed to be the steroid permease of this organism.

INTRODUCTION

Studies on transport processes in bacteria have mainly centred on characterizing the intact system in membrane vesicles prepared from the organism or in spheroplasts and intact cells. To further study the transport systems in depth, especially at a molecular level, it would be helpful if they could be extracted, their subcomponents purified and characterized, and then reconstituted in a synthetic system. There has been some success in this area lately in terms of using detergent extraction to isolate the lactose transport system from *Escherichia coli* [1, 2], an alanine carrier from *Bacillus subtilis* [3] and a proline carrier from *Mycobacterium phlei* [4]. A proline carrier was also extracted from *E. coli* with an organic solvent [5]. These were all shown to be transport systems for the respective substrates by being able to concentrate the substrate in synthetic phospholipid vesicles under the imposition of a potential gradient across the vesicle membrane.

The dicarboxylic acid transport system of *E. coli* was extracted with detergents and purified by affinity chromatography [6]. This sytem was characterized as consisting of two proteins which spanned the membrane with a substrate binding site on either side of the membrane. These proteins were shown to be transport proteins by their incorporation into rat myoblast and L-cell membranes which subsequently performed dicarboxylic acid transport which they had not done previously [7].

Pseudomonas testosteroni is a gram-negative bacterium capable of growing on certain C19 and C21 steroids in the absence of other suitable carbon sources. A steroid transport sytem has been identified in membrane vesicles prepared from this organism [8] and involves the 3β and 17β-hydroxysteroid dehydrogenase and the electron transport chain [9,10]. Steroid binding proteins have been located in the periplasmic space between the cytoplasmic membrane and the cell wall in the cell envelope of this organism [11]. Steroid binding proteins have also been found in loose association with the membrane vesicles [12–14]. Conventional classification of transport systems in bacteria is of the opinion that those systems which have periplasmic substrate binding proteins are sensitive to osmotic shock (which releases the periplasmic binding proteins) and do not survive in membrane vesicles. The steroid transport system of *P. testosteroni* is unusual in this respect because although this organism possesses a periplasmic steroid-binding protein, a steroid transport system does survive in membrane vesicles [8]. There is a precedent for this, however, since the dicarboxylic acid transport system of *E. coli* does have a periplasmic dicarboxylate binding protein and a transport system is found in isolated membrane vesicles [6].

To further study the steroid transport system of *P. testosteroni* we have attempted to isolate such a system from *P. testosteroni* membranes with an organic solvent and to reinsert it into synthetic phospholipid vesicles to examine steroid transport under the imposition of an artificial membrane potential. This paper reports the results of these studies.

EXPERIMENTAL

Organism

Pseudomonas testosteroni, ATCC 11996, was obtained from American Type Culture Collection, Rockville, Md.

Chemicals

Radioactive steroids, [^{14}C]amino acid labelling mixture, and 1, 4-*bis*(2(5-phenyloxazolyl)) benzene

(POPOP) were obtained from New England Nuclear, Boston, Mass., and unlabelled steroids from Steraloids Inc., Wilton, N. H. Ready-solv liquid scintillation cocktail was obtained from Beckman Instruments, Fullerton, Calif. Valinomycin, protease (Pronase E) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., St Louis, Mo., and 2,5-diphenyloxazole (PPO) from Fisher Scientific Co., Fairlawn, N.J.

Media conditions

Yeast extract medium supplemented with inorganic salts has been described previously [11]. Minimal medium contained per litre: ammonium dihydrogen phosphate, 1.0 g; diammonium hydrogen phosphate, 1.0 g; potassium dihydrogen phosphate, 2.0 g; magnesium sulfate heptahydrate, 0.2 g; sodium chloride, 0.01 g; 0.1 N sulfuric acid, 0.1 ml; zinc sulfate heptahydrate; 5 mg; manganese sulfate monohydrate, 4 mg; copper sulfate pentahydrate, 0.5 mg; and distilled water, 1000 ml.

Growth of cells and preparation of membrane extracts

Cells of *P. testosteroni* were grown in yeast extract medium for 12 h and harvested by centrifugation [8]. The cells were then washed in 30 mM potassium phosphate buffer, pH 7.0, and resuspended in the original culture volume of minimal medium supplemented with 0.5 g per l of testosterone and incubated for 16 h on a rotary shaker. The cells were then harvested by centrifugation.

In radiolabelling experiments the washed cells were incubated in minimal salts medium for 3 h to starve them. Testosterone was then added at a concentration of 0.5 g per l to induce the steroid transport system. Three h later 2 μCi [^{14}C]amino acid labelling mixture was added per l of culture and the cells were harvested by centrifugation after a further 7 h growth.

The harvested cells were washed twice in 30 mM potassium phosphate buffer, pH 7.0, suspended in this same buffer (1 g/3 ml) and broken by sonication. The broken cell suspension was centrifuged at 10,000 g for 15 min to remove unbroken cells and the supernatant was further centrifuged at 110,000 g for 1 h to precipitate the membranes.

To extract the steroid transport system the technique used was that reported by Amanuma and co-workers to extract the proline carrier from *E. coli* membranes [5]. The membrane pellet was washed twice with 30 mM potassium phosphate buffer, pH 7.0, and resuspended in distilled water (40–50 mg membrane/ml). To the membrane suspension was added 0.6 vol of *n*-butanol and 0.22 vol of pyridine adjusted to pH 4.3 with glacial acetic acid and the mixture was stirred for 40 min at 20°C. The mixture was then centrifuged at 24,000 g for 15 min and the butanol layer was removed, brought to dryness in a rotary evaporator at 30°C and stored *in vacuo* at 4°C until further use.

Phospholipids from whole cells of *P. testosteroni* grown on testosterone were extracted by the method of Folch [15].

Preparation of synthetic lipid vesicles

Synthetic lipid vesicles were constructed using the butanol–pyridine acetate extract and phospholipids extracted from *P. testosteroni*. The method was based on that reported by Amanuma *et al.* [5]. *P. testosteroni* phospholipids (80 mmol, as organic phosphate) and 1 mg of extracted and dried membrane protein were each placed separately in 1 ml of 0.1 M potassium phosphate buffer, pH 8.0, containing 0.1 M sodium chloride and sonicated in a bath type sonicator (Bransonic 12) for 1 min. The dispersed suspensions were then mixed together and sonicated for a further 2 min. The mixture was centrifuged at 5,000 g for 10 min to remove large aggregates and the milky supernatant was centrifuged at 110,000 g for 90 min to precipitate the potassium loaded vesicles. The pellet was washed with 2 ml of 0.2 M sodium chloride (adjusted to pH 8.0) with 10 mM sodium phosphate buffer and resuspended in 2 ml of this same sodium chloride solution. The suspension of synthetic vesicles was assayed immediately for steroid uptake.

Assays and analyses

The assay for steroid uptake by the synthetic liposomes was as follows. The reaction mixture contained in 50 μl: 20 μl of synthetic vesicles, 20 μl of 0.2 M sodium chloride and 173 pmol of radioactive steroid (635 dpm/pmol).

The reaction mixture was incubated for 3 min at 25°C. Then 0.5 nmol of valinomycin in 5 μl of 50% ethanol was added. At indicated times the reaction mixture was diluted with 5 ml of 0.2 M sodium chloride and filtered through 0.45 μ cellulose acetate membrane filters (Amicon, Lexington, Mass.) which were subsequently washed with another 5 ml of 0.2 M sodium chloride. The filters were dried and placed in 10 ml of PPO–POPOP scintillation cocktail and counted in a liquid scintillation spectrophotometer.

To assay for steroid-binding activity in the butanol–pyridine acetate extract the dried extract was dispersed by sonication in 10 mM potassium phosphate buffer, pH 6.0, containing 20% glycerol and 0.1% deoxycholate. The suspension was centrifuged at 10,000 g to remove solids and 1 ml samples of the supernatant were placed in dialysis bags and subjected to equilibrium dialysis. The samples were dialyzed for 20 h against a 15 ml solution of 10 mM potassium phosphate, pH 6.0, containing 20% glycerol, 0.1% deoxycholate, 5 μCi of radioactive and 1 μg of unlabelled steroid. After dialysis 0.2 ml of the material inside and outside the bag was dissolved in 10 ml of Ready-solv and counted in a liquid scintillation spectrophotometer. The difference between the counts inside and outside the bags was the amount of binding obtained.

Table 1. Binding of steroids by the butano–pyridine acetate extracted steroid binding activity*

Steroid	Binding activity (pmol/mg protein)
C19 steroids:	
Testosterone	62.2
Androstenedione	39.8
C21 steroids:	
Corticosterone	0
Progesterone	49.8
C18 steroids:	
Estradiol-17β	0

*Binding assays were performed on the dried extract which had been resolubilized by sonication in 10 mM potassium phosphate buffer containing 20% glycerol and 0.1% deoxycholate as outlined in Experimental.

Lipid concentration was determined as organic phosphate by the method reported by Ames and Dubin [16]. Protein was assayed by the Coomassie blue technique reported by Bradford [17] using bovine serum albumin as a standard. SDS-gel electrophoresis was performed by the technique of Laemmli [18]. Gels were stained with silver nitrate [19].

For protease treatment, the butanol–pyridine acetate extract was solubilized in 10 mM potassium phosphate buffer, pH 7.0 containing 20% glycerol and 0.1% deoxycholate. Then 50 μg of Protease was added per mg of membrane protein for 10 min at 25°C. The reaction was terminated by adding the enzyme inhibitor, PMSF, to a final concentration of 0.1 mM and the solution was then assayed for binding activity in the presence of 0.1 mM PMSF. Controls for steroid binding by the protease were performed by treating samples of protease alone in the above manner and assaying for binding activity.

RESULTS

Steroid binding activity in the butanol–pyridine acetate extract

When the dried extract was solubilized in 10 mM potassium phosphate buffer containing 20% glycerol and 0.1% deoxycholate there was some steroid binding activity present. This activity bound the C19 steroids andostenedione and testosterone and the C21 steroid progesterone (Table 1). It did not bind the C18 steroid estradiol or the C21 steroid corticosterone. Binding of testosterone was saturable and from a Scatchard plot of binding at different testosterone concentrations and affinity constant, K_d, was calculated to be 6.35 nM (Fig. 1). Binding of radiolabelled testosterone was displaced by excess unlabelled testosterone but only partially (60%) destroyed by treatment with protease.

Incorporation of binding activity into liposomes

Liposomes constructed from *P. testosteroni* lipids and the butanol–pyridine acetate extract were first tested for intactness by examining them in hypotonic solution under phase contrast microscopy. The liposomes were observed to increase in size as they absorbed water until they burst indicating that they were, in fact, intact entitites.

Electron microscopy of the synthetic liposomes revealed structures of two types: multilamellar structures which were very large (Fig. 2a) and small vesicles which showed double track membranes in thin sections and in freeze fractures showed protein studs protruding from off the inner track of the membrane (Fig. 2b).

Steroid uptake by liposomes

Assays of steroid uptake by the synthetic liposomes in the presence of the ionophore valinomycin showed that testosterone was taken up but not androstenedine. Uptake reached a maximum at 30 s and then declined (Fig. 3). Uptake was dependent upon the presence of liposomes containing the butanol–pyridine extract from cells of *P. testosteroni* grown on testosterone. It was also dependent on the presence of potassium phosphate inside the vesicles and the ionophore valinomycin. There was no uptake if the vesicles were preloaded with sodium phosphate instead of potassium phosphate.

Composition of the butanol–pyridine acetate extract

The organic solvent extract contained both protein and lipid and reproducible electrophoretic results could not be obtained unless the lipids were removed. This could be achieved by passing the concentrated extract through a small (1.0 × 20 cm column of Sephadex G-25 equilibrated with 30 mM potassium phosphate buffer, pH 6.0, containing 20% glycerol and 0.1% deoxycholate. The proteins were not retained by the column and were eluted in the void volume while the lipids were retarded by the column. SDS-gel electrophoresis of the protein fraction revealed the presence of six major protein bands (Fig. 4). A comparison of the banding pattern in the extracts of induced vs uninduced cells showed that two protein bands were present in the induced cells

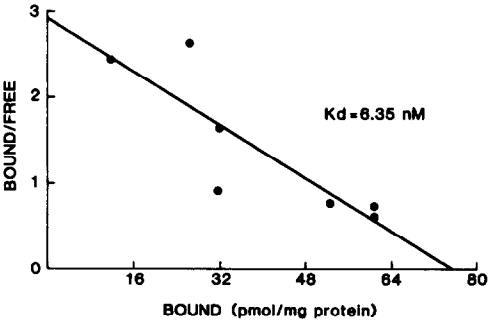

Fig. 1. A Scatchard plot of testosterone binding by buranol–pyridine acetate extract resolubilized in 10 mM potassium phosphate buffer, pH 6.0, containing 20% glycerol and 0.1% deoxycholate. The coefficient of correlation was 0.75.

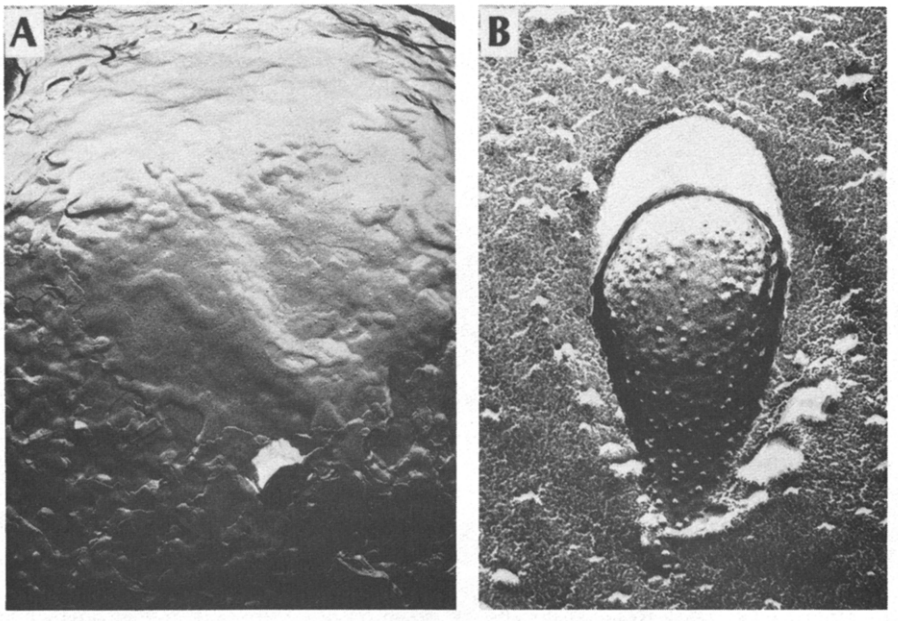

Fig. 2. Electron micrographs of freeze fracture preparations of synthetic vesicles constructed from the butanol–pyridine acetate extract and *P. testosteroni* phospholipids. A: A large multilamellar vesicle (×32,000). B: A smaller vesicle showing protein studs extending from the inner lipid layer of the membrane ×80,000.

which were absent in the uninduced cells. These protein bands had molecular weights of 56,000 and 45,000.

Adding [^{14}C]amino acids labelling mixture to cells growing on testosterone allowed incorporation of the label into the cell membrane fraction (Table 2) and some of this could be traced to the butanol–pyridine acetate extract. Passage of the extract through the Sephadex G-25 column removed virtually all the radioactivity from the protein fraction indicating that the label was present in lipid rather than in protein. By washing the total extract with chloroform–methanol (2:1, v/v) to remove lipids the label was removed into the organic wash. Thin layer chromatography of this wash fraction with lipid standards revealed that the radioactivity was present in phosphatidylethanolamine. This was further confirmed by extracting the radioactive spot and subjecting it and an authentic sample of phosphatidylethanolamine to hydrolysis. The hydrolysis products were identical in their migratory behavior on thin layer chromatography in different solvent systems. It was thus concluded that the radioactive label had been incorporated into the ethanolamine moiety of the phosphatidylethanolamine molecule.

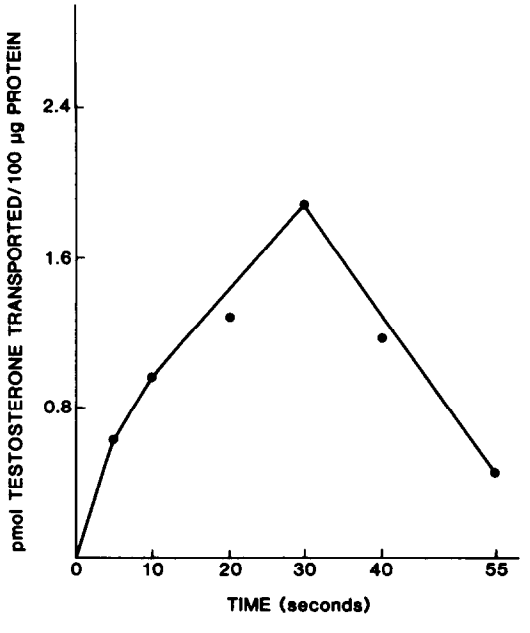

Fig. 3. Testosterone uptake by the synthetic lipid vesicles in the presence of valinomycin. The conditions for the uptake assay are outlined in Experimental.

DISCUSSION

A steroid transport system has been extracted from *P. testosteroni* membranes and incorporated into synthetic lipid vesicles constructed from the phospholipids of this same organism. Uptake is dependent upon the presence of vesicles constructed from the butanol–pyridine acetate extract and preloaded with potassium phosphate, the ionophore valinomycin and the substrate testosterone. Since only testosterone is transported this must be the transport system specific for this steroid. The ionophore valinomycin is believed to function by causing leakage

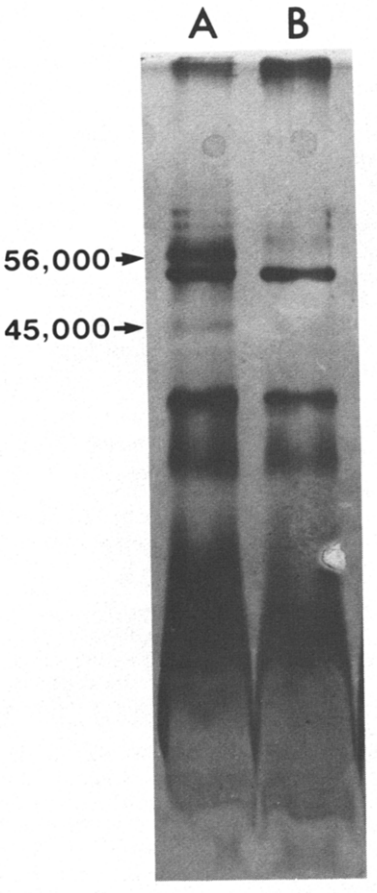

Fig. 4. SDS-gel electrophoresis of the proteins present in the butanol–pyridine acetate extract of *P. testosteroni* membranes after elution from the Sephadex G-25 column. Electrophoresis was performed by the technique of Laemmli using 12% arcylamide gels which were subsequently stained with silver nitrate. Lane A: the extract of induced cells; Lane B: the extract of uninduced cells grown in the absence of testosterone. The arrows indicate the protein hands present in the induced cell membranes only.

Trivial and sytematic nomenclature of steroids: Testosterone: 17β-hydroxy-4-androsten-3-one. Androstenedione: 4-androstene-3, 17-dione. Corticosterone: 21-hydroxy-4-pregne-3,20-dione. Progesterone: 4-pregnene-3,20-dione. Estradiol 17: 1,3,5 (10)-estratriene-3, 17β-diol.

of potassium ions from inside the vesicles creating a membrane potential whereby the insides of the vesicles are negatively charged with respect to the outside of the vesicles. This membrane potential drives the uptake of testosterone.

The uptake process was maximum at 30 s after which time the uptake ability was quickly lost. This is probably due to dissipation of the membrane potential as the concentration of potassium equilibrated across the membrane of the liposomes. It is unlikely that this is a binding rather than an uptake process since it is inhibited by the presence of sodium instead of potassium inside the liposomes. Because valinomycin is not an effective ionophore for sodium,

Table 2. Distribution of [^{14}C]amino acid label in cell culture and subcellular fractions

Fraction*	Total cpm/l of culture
Cell culture	2.4×10^6
Culture supernatant	5×10^5
Cells only	1.9×10^6
Cell disruption:	
Membrane fraction	5×10^5
High speed centrifugation:	
Membrane pellet	2×10^5
Supernatant	3×10^5
Butanol pyrindine extract of membrane pellet	1.4×10^5

*The steps and procedures used to generate the culture and subcellular fractions are as explained in Experimental.

a membrane potential is not created under these conditions and testosterone uptake is not achieved.

The steroid-binding activity present in the butanol–pyridine acetate extract bound testosterone with high affinity. Protease destruction of binding indicates that the binding activity is, at least partially, composed of protein. It is unusual that protease treatment did not destroy all the binding since the activity of other soluble steroid binding proteins from *P. testosteroni* is totally destroyed under identical conditions (unreported results). However, since the extract contains lipids, these may partially protect the proteins from protease attack.

Since the steroid transport system was present in the extract of cells grown on testosterone but absent in that of uninduced cells, one of the two additional protein bands present in the induced extract may be the steroid permease or both protein bands may work together to perform this function. Either possibility is feasible since while the lactose permease of *E. coli* is one protein species [1], that of the dicarboxylate transport system in *E. coli* is composed of two different proteins [6, 7].

Using the [^{14}C]amino acid labelling mixture to incorporate radioactivity into the membrane proteins did result in labelling of the membrane faction. However, the majority of the radiolabel was present in phosphatidylethanolamine of the lipid component of the membrane and is probably due to direct incorporation of serine into phosphatidylserine which is the precursors of both phosphatidylethanolamine and phosphatidylcholine. Therefore, these latter two lipids should have been labelled also. However, since phosphatoadylethanolamine is the major lipid in this *Pseudomonas* species it is not unusual for the label to be concentrated in this lipid molecule.

The steroid transport system extracted by the organic solvent system appears to be specific for testosterone since androstenedione is not transported. That system present in isolated membrane vesicles of *P. testosteroni* transports testosterone but not androstenedione [9] which is the intravesicular steroid [9]. It has been proposed that in the membrane vesicle system, during the transport process, testosterone is converted to androstenedione by the 3β and 17β-hydroxysteroid dehydrogenase which has

been localized on the cytoplasmic membrane [20]. The electrons derived from this reaction are passed down the electron transport chain via reoxidation of NADH by NADH dehydrogenase with the subsequent extrusion of protons thereby providing a membrane potential which would drive the transport process. Since the 3β and 17β-hydroxysteroid dehydrogenase is located on the external vesicle surface, and androstenedione is the intravesicular steroid, this must be the form in which the steroid is transported. It is possible that there is more than one steroid transport system operating by different mechanisms in *P. testosteroni* and that which survives in isolated membrane vesicles is different from the one extracted by the organic solvent system.

There are steroid binding proteins loosely associated with the cytoplasmic membrane of *P. testosteroni* [12–14] and these have different specificities for different steroids. Perhaps through their interaction with a steroid permease they confer a specificity to this molecule. Ames and co-workers have identified a membrane component of the histidine and arginine transport systems of *Salmonella typhimurium* which interacts with the periplasmic binding proteins for these substrates and apparently achieves substrate specificity by this interaction [21, 22]. An analogous condition may be operative in the steroid transport system of *P. testosteroni* whereby the membrane-assocaited steroid binding proteins may change the specificity of the steroid transport protein. Experiments are currently underway to investigate this possibility.

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