Acta Chemica Scandinavica B 38 (1984) 433–438

Partial Characterization of an Androgen-induced Androgen-binding Protein in *Pseudomonas Testosteroni*

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An androgen-induced protein in *Pseudomonas testosteroni* binding [^{3}H]androstenedione, [^{3}H]testosterone and [^{3}H]5α-dihydrotestosterone but not estradiol or corticosterone with high affinity (K_d=2×10^{-9} M) is partially characterized. The [^{3}H]androgen-protein complex forms a 3.5 S peak when analyzed on sucrose gradients, a peak corresponding to a Stokes radius of 26 Å on Sephadex G-150 chromatography, and is partly (15 %) bound to DNA-cellulose.

The mechanism of androgen action in *P. testosteroni* has not been characterized although it is well known that specific enzymes are induced by androgens. As this androgen-binding protein shares many of the characteristics described as typical for androgen receptors, this macromolecule may mediate androgen action in *P. testosteroni*. This suggests a possible use of this microorganism as a model system to study androgen action. It is tempting to speculate that these cells can be used as source for purification of an androgen receptor complex.

Pseudomonas testosteroni is a microorganism capable of utilizing steroids as its sole carbon source for growth. The enzymes involved in the metabolism of steroids by *P. testosteroni* are induced by steroids, notably androgens.[1] The physiological role of androgens as well as several aspects on their mechanism of action, mediated *via* receptor proteins in target tissues, is well known in mammalian species.[2] However, the corresponding function of androgens in *P. testosteroni* is rather obscure, despite their well known enzyme-inducing capacity. Efforts to find steroid receptor proteins in bacteria have proved unsuccessful, and it has been suggested that the effects of androgens upon microorganisms might be mediated *via* another mechanism not including receptor proteins.[3] However, other groups have reported on the presence of androgen binding "receptor proteins" in P. *testosteroni*[4] and *Streptomyces hydrogenans*.[5] During our studies on androgen receptor mechanisms in mammalian tissues we have made efforts to find a suitable model system and a source for purification of androgen-receptor complex. In view of the reported results of Watanabe and collaborators[4] we have focused our interest upon *P. testosteroni*. The present investigation was undertaken to characterize an androgen-binding protein in this microorganism.

MATERIALS AND METHODS

Steroids. [1,2,6,7-^{3}H]Testosterone (specific radioactivity 3100 GBq/mmol), [1,2,6,7-^{3}H]4-androstene-3,17-dione (specific radioactivity 3300 GBq/mmol), [1,2,4,5,6,7-^{3}H]5α-dihydrotestosterone (specific radioactivity 3700 GBq/mmol), [2,4,6,7-^{3}H]estradiol-17β (specific radioactivity 3360 GBq/mmol) and [1,2,6,7-^{3}H]corticosterone (specific radioactivity 3000 GBq/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled testosterone, 5α-dihydrotestosterone, androstenedione, estradiol-17β and corticosterone were purchased from Sigma Chemical Co., St. Louis, MO, USA. Cyproterone acetate was obtained from Schering AG, Berlin, W. Germany.

Growth of organism and preparation of cell-free extracts. P. testosteroni ATCC 11996 was obtained from the American Type Culture Col-

0302-4369/84 $2.50

lection, Rockville, Md., USA. It was grown on a medium containing 1 g $NH_4H_2PO_4$, 1 g $(NH_4)_2HPO_4$, 2 g KH_2PO_4, 10 g Difco yeast extract, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 mg NaCl, 5 mg $ZnSO_4 \cdot 7H_2O$, 5 mg $MnSO_4 \cdot 3H_2O$, 0.5 mg $CuSO_4 \cdot 5H_2O$ and 0.1 ml H_2SO_4 per 1, adjusted to pH 6.65 (4). The organism was grown on a shaking table at 28 °C. Addition of inducer steroid in ethanol was carried out 48 h after inoculation and the cells were harvested after a total growth period of 4 d. The cultures were centrifuged in a refrigerated centrifuge at 800×*g* for 10 min and the cells were washed twice with ice-cold 0.15 M NaCl in 50 mM Tris-HCl, pH 9.0.

Cell-free extracts were prepared by osmotic shock or by sonication alternatively. All operations were carried out at 0–4 °C unless otherwise stated. For osmotic shock treatment the cells were resuspended in 40 ml of 30 % (w/v) sucrose in 50 mM Tris-HCl, pH 9.0, containing 5 mM EDTA per g wet weight. The suspension was incubated at 25 °C for 60 min and centrifuged at 8000×*g* for 30 min. The cells were resuspended in a small volume of 30 % sucrose, rapidly dispersed in the original volume of icecold buffer A (1 mM Tris-HCl, pH 9.0, containing 0.5 mM $MgCl_2$ and 1 mM dithiothreitol) and stirred for 30 min at 4 °C, after which the cell debris was removed by centrifugation at 25 000×*g* for 30 min. The cell-free extract was concentrated 20-fold by ultrafiltration.

For sonication the washed cells were resuspended in buffer A and sonicated for 2×20 s. The extract was purified as described above. Streptomycin sulfate was added to the concentrated cell-free extract to a final concentration of 1 % (w/v) and the precipitated DNA removed by centrifugation. The supernatant was dialyzed against 100 volumes of 50 mM Tris, pH 9.0, 5 mM EDTA and 1 mM dithiothreitol for 16 h. The dialyzed supernatant was stored at −20 °C until further experiments were performed.

Steroid binding assay. Aliquots of the cell extract were incubated for 16 h at 0–4 °C with different amounts of labelled and unlabelled steroids. Free steroids and steroids bound to macromolecules were separated on Sephadex G-25 equilibrated in buffer A.

As an alternative method, free and macromolecule-associated steroids were separated by treatment with dextran-coated charcoal (dcc). Three volumes of cell extract were mixed with 1 volume of dcc suspension (0.5 % (w/v) dextran, 0.05 % (w/v) charcoal) and the incubation mixture was allowed to stand for 10 min before the charcoal was removed by centrifugation. Radioactivity measurements were performed using Instagel (Packard Instrument Co. Inc., Warrenville, Downess Grove, Ill., USA) as scintillation liquid. Radioactivity was measured as dpm using the external standard technique. Specific binding was calculated as the difference between radioactive steroid bound in the absence (total binding) and in the presence (nonspecific binding) of 100 times excess of unlabelled steroid.

Density gradient centrifugation. Aliquots (0.2 ml) of samples containing macromolecule-bound steroid were layered on top of linear 5 ml 5–20 % (w/v) sucrose gradients in buffer A, 0 or 0.4 M with respect to KCl. The tubes were centrifuged for 15 h at 45000 rpm using an SW 50.1 rotor. At the end of the centrifugation, the bottom of the tube was punctured and 3–4 drop fractions were collected and measured for radioactivity. The following markers were used: bovine serum albumin (4.6 S) and cytochrome c (1.73 S).

Sephadex G-150 chromatography. Sephadex G-150 was purchased from Pharmacia, Uppsala, Sweden. In most cases unfractionated incubation mixtures were applied on the column. The following calibration standards were used: dextran blue, alcohol dehydrogenase (Stokes radius (SR) 46), bovine serum albumin (SR 35), hemoglobin (SR 24) and cytochrome c (SR 17). The Stokes radius of the androgen binding protein was calculated as described by Siegel and Monty.[6]

Other procedures. Ammonium sulfate precipitation, DEAE-cellulose chromatography, DNA-cellulose chromatography and protease treatment were performed using the protein-bound fraction after filtration through a Sephadex G-25 column equilibrated in TE-buffer (50 mM Tris-HCl, pH 9.0, 5 mM EDTA).

Ammonium sulfate was added to yield final concentrations of 20 %, 40 % and 60 % of saturation. Precipitates were removed by centrifugation and redissolved in buffer before aliquots were taken for measurements of radioactivity.

DEAE-cellulose chromatography was performed using a discontinuous gradient of 0.005, 0.01, 0.02, 0.05, 0.07, 1.0 and 2.0 M KCl in TE-buffer.

DNA-cellulose chromatography was carried out as described by Mainwaring *et al.* [7]. An aliquot of the sample was also chromatographed on a cellulose column to define nonspecific binding. The samples were applied in TE-buffer and the columns were washed with the same buffer until no more radioactivity was eluted. The columns were then eluted with TE-buffer, 0.4 M with respect to KCl.

In order to investigate the nature of the androgen-binding macromolecule, aliquots of the protein-bound fraction were treated with pro-

tease, 1 mg/ml, type VI (Sigma Chemical Co., St. Louis, Mo., USA) for 4 h at 20 °C. The remaining androgen–macromolecule complex was measured after filtration through a Sephadex G-25 column. Aliquots not treated with protease were run in parallel in order to measure the spontaneous degradation of the complex.

In selected cases aliquots were taken for identification of radioactive metabolites. Steroids were extracted with chloroform:methanol (1:1, v/v) and the chloroform phases were evaporated to dryness. Steroids were identified by thin-layer chromatography and, after silylation, by radiogas chromatography[8]. A steroid was considered as identified if it had the same thin-layer chromatographic mobility and the same retention time on OV-17 as the reference steroid.

RESULTS

Results on specific binding of androgen in extracts from *P. testosteroni,* analyzed according to Scatchard [9], showed a K_d of 2.0×10^{-9} M and an amount of binding sites of 2×10^{-12} mol/mg of protein when [^{3}H]androstenedione was used as ligand (data not shown). As shown in Fig. 1a, both [^{3}H]androstenedione, [^{3}H]testosterone and [^{3}H]5α-dihydrotestosterone formed macromolecule complexes sedimenting at 3.5 S, whereas [^{3}H]estradiol and [^{3}H]corticosterone did not bind to macromolecules sedimenting in the 3–4 S region. The 3.5 S [^{3}H]androstenedione-macromolecule complex was totally displaced by a 100-fold excess of unlabelled androstenedione (Fig. 1b), testosterone or 5α-dihydrotestosterone. Corticosterone and estradiol did not compete for the androgen-binding sites (Fig. 1a). The [^{3}H]androgen-macromolecule complex sedimented at 3.5 S both at low and high ionic strength. Thin-layer chromatography and radiogas chromatography showed that the androgen bound to the macromolecule was identical to the incubated androgen. No specific binding of androgen was observed in cell extracts from uninduced cultures.

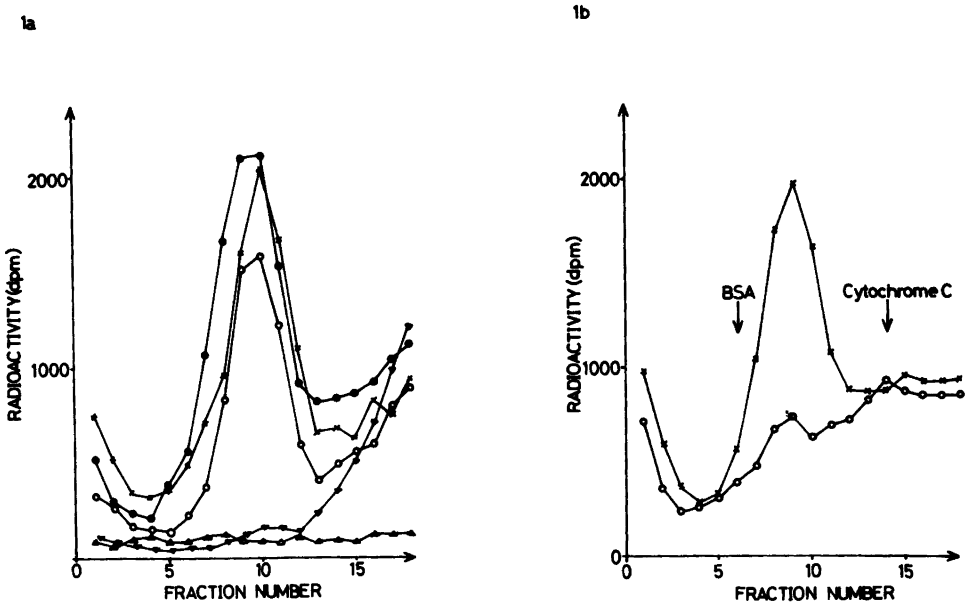

Fig. 1. Aliquots of cell extracts (protein concentration 1 mg/ml) were incubated with 10^{-9} M [^{3}H]labelled steroid for 16 h and, after filtration through Sephadex G-25, 0.2 ml of the macromolecule-bound fraction was analyzed by density gradient centrifugation. The tubes were centrifuged at 50,000 r.p.m. for 24 h using an SW 50.1 rotor.

1a. Incubation of 10^{-9} M [^{3}H]androstenedione, ×; [^{3}H]testosterone, ○; [^{3}H]5α-dihydrotestosterone, ●; [^{3}H]estradiol, ▼; [^{3}H]corticosterone, △.

1b. Incubation of 10^{-9} M [^{3}H]androstenedione, ×; [^{3}H]androstenedione+unlabelled androstenedione, ○.

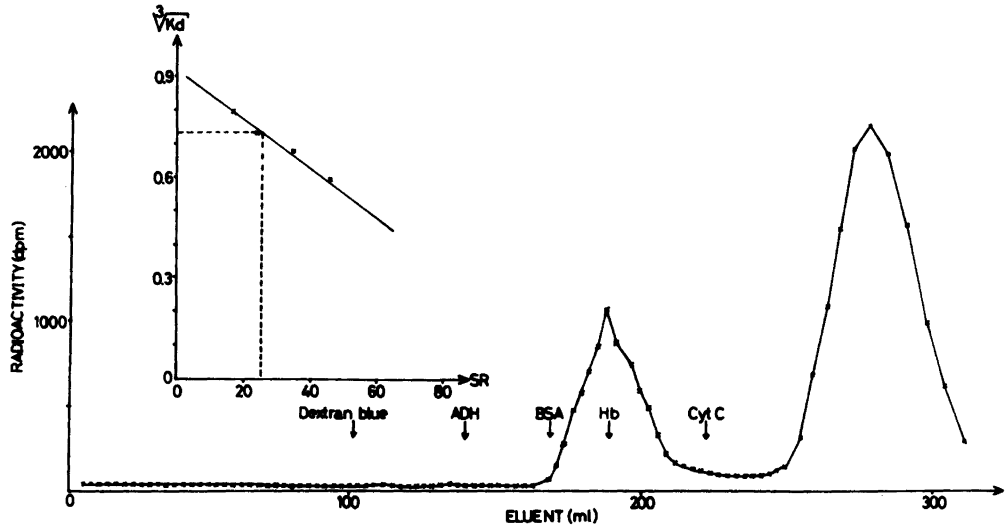

Fig. 2. Cell-free extract from *P. testosteroni* (protein concentration 1 mg/ml) was incubated with 10^{-9} M [^{3}H]androstenedione for 16 h. Three ml of the incubation mixture was applied on a Sephadex G-150 column that was eluted with buffer A. The following standards were used: dextran blue (void volume), alcohol dehydrogenase (SR 46), bovine serum albumin (SR 35), hemoglobin (SR 24) and cytochrome c (SR 17).

Chromatography of the [^{3}H]androstenedione-macromolecule complex on Sephadex G-150 was performed in buffer A with or without 0.2 M KCl. In both cases, the complex appeared to have a Stokes radius of 26 Å (Fig. 2). The steroid bound in the eluted complex was identified as [^{3}H]androstenedione. Ammonium sulfate fractionation indicated that the [^{3}H]androstenedione-macromolecule complex was precipitated between 20 and 40 % saturation (Fig. 3). When chromatographed on a DEAE-cellulose column the [^{3}H]androstenedione-macromolecule complex was eluted with 0.01 M KCl. Chromatography on DNA-cellulose showed that 15 % of the applied macromolecule-associated [^{3}H]androstenedione was retained on the column at low ionic strength and was eluted with 0.4 M KCl. No radioactivity was retained on cellulose columns.

Protease treatment destroyed 75 % of the [^{3}H]androstenedione-macromolecule complex.

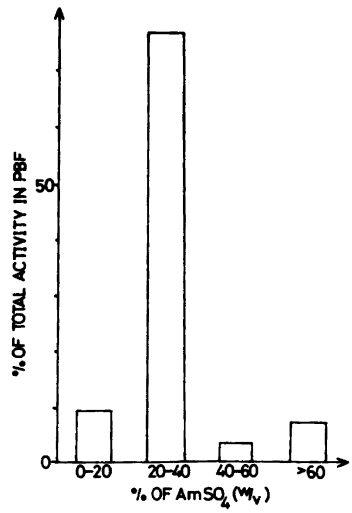

Fig. 3. Cell-free extract from *P. testosteroni* (protein concentration 1 mg/ml) was incubated with 10^{-9} M [^{3}H]androstenedione for 16 h. After filtration through a Sephadex G-25 column the protein-bound fraction (PBF) was subjected to ammonium sulfate fractionation.

DISCUSSION

According to the current concept of steroid mechanism of action, the steroid hormone, following passage through the cell membrane, is

bound to a cytoplasmic receptor protein. The steroid-receptor complex is transferred into the cell nucleus, where it interferes with the chromatin and induces the synthesis of specific mRNA which are transferred to the polysomes and code for the synthesis of specific induced proteins. Specific steroid receptor proteins have been described in most steroidsensitive tissues (2).

In *P. testosteroni*, Δ^1-dehydrogenase-, 3β- and 17β-hydroxysteroid dehydrogenase and especially 3-keto-Δ^5-steroid isomerase activities are induced by steroids, notably androgens.[1] The mechanism by which androgens induce the enzyme activities is still obscure. Wacher *et al.* [10] showed an effect of testosterone on the activity of DNA-dependent RNA-polymerase in *P. testosterone*, and suggested that a testosterone receptor protein might act as a repressor on the synthesis of RNA-polymerase. Mainwaring *et al.*[3] studied binding of [^{3}H]androgens to cell extracts from various bacteria, including induced and non-induced cultures of *P. testosteroni* and found no binding to receptor molecules either with sucrose density gradient centrifugation or gel-exclusion chromatography. In a number of papers, Watanabe *et al.*[4,11,12] have described the transport and proteinbinding of steroids in *P. testosteroni*. Special interest was focused on a protein binding [^{3}H]androgens that was only formed in induced cultures and sedimented at 3.5 S when analyzed by sucrose gradient centrifugation.

This paper confirms the presence of a 3.5 S androgen binding protein in *P. testosteroni* that binds [^{3}H]androgens with high affinity. Furthermore, we have partially characterized the complex formed between this protein and [^{3}H]androstenedione by chromatography on DEAE-cellulose, DNA-cellulose and Sephadex G-150 and by fractionation with ammonium sulfate. The physiological role of this protein can not be stated at the present time. Different steroid binding proteins are present in steroid target cells: Receptor proteins characterized by binding to the ligand with high affinity and showing high ligand specificity (2,13); other steroid binding proteins with rather high affinity and specificity probably acting as steroid-transporting proteins (14,15); enzymes, showing lower affinity and ligand specificity and so called unspecific binding proteins, *e.g.* extracellular albumin. The protein described in this paper can not be directly classified, but the high affinity (K_d 2×10^{-9} M) and especially the binding to DNA-cellulose, which is in the same range as found using prostatic androgen receptor,[16] indicates a relationship with receptor proteins. On the other hand, the ability to bind androstenedione is not shared with most other androgen receptor proteins which bind testosterone and 5α-dihydrotestosterone with higher affinity (2,13,17). However, a receptor protein that binds androstenedione has been demonstrated in rat liver[18]. The precipitation of the bacterial receptor protein between 20–40 % (w/v) of ammonium sulfate and the sedimentation of the androgen-protein complex at 3.5 S are characteristics found for most androgen-receptor complexes.[2]

Maximal binding was calculated to about 10^{-12} mol/mg of cytosol protein which is probably an underestimation as unmetabolized unlabelled androgens might remain following induction. This amount is higher than reported for most androgen receptor proteins but similar to what has been found for gluco-corticoid and estradiol receptors. It is therefore easy to believe that a, in some way, very specialized cell may show higher amounts than present in a prostatic cell.

Although the androgen-binding protein in extracts from *P. testosteroni* appeared to be devoid of steroid-metabolizing capacity, it cannot be totally excluded that this protein acts as an enzyme.

No androgen-binding activity was detected in uninduced cultures of *P. testosteroni* indicating either absence or very low amounts of receptor. In some tissues steroids seem to induce the synthesis of their own receptors [2], and if this is the case with *P. testosteroni* androgen receptors may be present in minor amounts in uninduced bacterial cultures.

Taken together the characteristics of this protein are similar to what could be expected of a specific androgen binding transport or receptor protein, which following formation of an androgen-macromolecule complex, interacts with the DNA-molecule and induces specific mRNA-molecules. Therefore it is quite possible that this protein serves as a receptor protein and mediates androgen activity in this microorganism. That would indicate that androgens act in a similar way in this microorganism as in classical androgen target tissues, and that *P. testosteroni* can be used as a model system to study the mechanism of action of androgens. Moreover, it is tempting to

speculate that this protein has remained rather unchanged through the evolution and therefore should be immunologically similar to mammalian androgen receptors. If so this microorganism could be a good source for purification of the androgen receptor.

Acknowledgements. This work was supported by a grant from the Swedish Medical Research Council.

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Received August 17, 1983.