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BINDING OF STEROIDS BY A PARTIALLY PURIFIED PERIPLASMIC PROTEIN FROM *PSEUDOMONAS TESTOSTERONI*

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SUMMARY

The androgen-binding periplasmic protein of *Pseudomonas testosteroni* was released by lysozyme-EDTA treatment of induced bacteria and partially purified by ammonium sulfate fractionation. The androgen-binding protein appeared in the 50–80% saturated fraction whereas steroid transforming enzymes and the estradiol-binding protein appeared in the 0–50% saturated fraction. The partial purification, however, did not alter the substrate specificity of the androgen-binding protein. Kinetics of binding at this stage of purification indicated a simple equilibrium binding process with independent binding sites.

INTRODUCTION

In gram-negative bacteria, active transport mechanisms may be divided into those which are sensitive or those insensitive to osmotic shock treatment. The osmotic shock insensitive system can be studied in isolated membrane vesicles and is stimulated by respiratory chain dependent oxidation of D-lactate and other electron donors [1, 2]. Recently, we have reported a transport system for steroids using membrane vesicles of *Pseudomonas testosteroni* which appear to have characteristics of both group translocation [3, 4] as well as an active transport system requiring both the electron transport chain [5] and the enzyme 3β- and 17β-hydroxysteroid dehydrogenase [4, 6].

Transport systems sensitive to osmotic shock treatment are related to solute-binding proteins located in the periplasmic space, the space between the inner and outer membranes which constitute the cell envelope in gram-negative organisms. In *P. testosteroni* we have reported the presence of an inducible steroid-binding activity possessing characteristics of a protein molecule, and releasable from bacterial cells by cold osmotic shock treatment [7]. This steroid-binding activity appears to be significant to the growth of this organism on steroid since there is a correlation between binding of various steroids and the ability of this organism to grow on such steroids with induction of degradative enzymes and steroid binding activity [8]. Furthermore, inhibition of steroid binding occurs at 37 and 45°C, temperatures at which the organism will not grow on steroids nor induce binding activity [8].

Although we have reported some of the characteristics of the periplasmic steroid-binding protein [7, 9], we have not systematically examined the requirements of the binding reaction. In this paper, we report on the release of steroid-binding protein by lysozyme–EDTA treatment, and the characteristics and requirements for binding of the partially purified periplasmic protein.

EXPERIMENTAL PROCEDURES

Materials

P. testosteroni 11996 was obtained from American Type Culture Collection, Rockville, Maryland; unlabelled testosterone* from Steraloids, Pawling, New York; [7-^{3}H]-testosterone (25 Ci/mmol), [1,2-^{3}H]-androstenedione (48 Ci/mmol), and [6,7-^{3}H]-estradiol-17β (46.6 Ci/mmol) from New England Nuclear Corporation, Boston, Massachusetts.

Methods

Growth conditions and induction of binding activity. Media used for growth and conditions for induction of binding activity have been described [7]. Testosterone was added to a final concentration of 0.5 g/l. for induction of binding activity and induction period was 40 h.

* Trivial and systematic nomenclature of steroids: Testosterone, 17β-hydroxy-4-androsten-3-one; androstenedione, 4-androstene-3,17-dione; estradiol-17β, 1,3,5(10)-estratriene-3,17β-diol; androsterone, 3α-hydroxy-5α-androstan-17-one; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; 5α-androstanedione, 5α-androstane-3,17-dione; 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; epitestosterone, 17α-hydroxy-4-androsten-3-one; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; progesterone, 4-pregnene-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; cortisol, 11β-17,21-trihydroxy-4-pregnene-3,20-dione.

Table 1. Release of androgen binding protein by osmotic shock and lysozyme EDTA treatment of induced *P. testosteroni*

Experiment	Total protein		Binding activity			Specific activity	
	OS	LE	OS	LE	Total	OS	LE
	(mg)		(pmol steroid bound)			(pmol/mg protein)	
1	640	38	992	60	1052	1.55	1.59
2	840	1340	210	4623	4833	0.25	3.45
3	1340	2660	0	6410	6410	0	2.41
4	640	1100	358	660	1018	0.56	0.60
5	1720	2960	1410	3552	4962	0.82	1.20
6	1100	1460	0	1255	1255	0	0.86
7	-	1000	-	4440	4440	-	4.44

Note 1. Binding protein was prepared from cells by osmotic shock and/or lysozyme–EDTA treatment as described in the text. The lysozyme–EDTA supernatant was concentrated by ammonium sulfate precipitation (0–80%) prior to assaying for binding activity. In Experiment 7 the cells were not subjected to osmotic shock prior to lysozyme–EDTA treatment.

Note 2. Binding of steroids was determined by equilibrium dialysis. Periplasmic binding protein, at two levels of protein concentration in a total volume of 1 ml, was equilibrated with 15 ml of dialyzing buffer containing 5 mM EDTA, 0.5 mM dithiothreitol, 2 nM [1.2-^{3}H]-androstenedione (2.3×10^7 c.p.m./nmol) and 50 mM Tris–HCl, pH 9.0, at 4°C for 20 h. Tritium present inside and outside the bag was determined in a liquid scintillation spectrometer.

Note 3. OS = osmotic shock treatment; LE = lysozyme–EDTA treatment.

Preparation of osmotic shock protein. Osmotic shock protein was prepared as previously described [7] with minor modifications. Bacterial cells were grown at 30°C to stationary phase and induced by the addition of testosterone to a final concentration of 0.5 g/l. In most experiments, cells were harvested by centrifugation at 8000 *g* for 10 min at 4°C and washed twice with 0.15 M NaCl, 50 mM Tris–HCl, pH 9.0. In Experiments 5–7, Table 1, NaCl and Tris–HCl were added before harvesting and the cells were not subsequently washed. Each g of wet cells was resuspended, with stirring, in 40 ml of 30% (w/v) sucrose, 50 mM Tris–HCl, pH 9.0, and EDTA was added to a final concentration of 5 mM. The mixture was incubated in a rotatory shaker at 25°C for 20 min and the cells were harvested by centrifugation at 8000 *g* for 20 min. The cells were resuspended in a small volume of 30% sucrose, 50 mM Tris–HCl, pH 9.0, rapidly dispersed in an original volume of ice-cold buffer (1 mM Tris–HCl, pH 9.0, 0.5 mM $MgCl_2$ and 1 mM dithiothreitol) and gently stirred for 25 min at 4°C. Cells were then removed by centrifugation at 8000 *g* for 10 min. The supernatant thus obtained was concentrated 10-fold by ultrafiltration through an Amicon DC-2 concentrator. The supernatant was dialyzed against 100 vol. of 1 mM Tris–HCl, pH 9.0, containing 0.5 mM $MgCl_2$ and 1 mM dithiothreitol, for 20 h at 4°C. This is referred to as the osmotic shock protein.

Release of steroid-binding protein by lysozyme–EDTA treatment. Depending on the experiment, cells harvested following osmotic shock treatment or cells not previously exposed to osmotic shock were treated with lysozyme–EDTA. Each g of wet cells was suspended in 80 ml of 20% sucrose, 30 mM Tris–HCl, pH 9.0, at 25°C with stirring. After 30 min, EDTA and lysozyme were added to final concentrations of 10 mM and 0.5 mg/ml, respectively, and the suspension was incubated with stirring for a further 30 min at 25°C. The suspension was centrifuged at 8000 *g* for 20 min. The supernatant thus obtained, containing the periplasmic binding protein, was referred to as the periplasmic fluid or lysozyme–EDTA supernatant. In the experiments described in Tables 1–4, the lysozyme–EDTA supernatant was concentrated by ammonium sulfate precipitation, while in all other experiments it was concentrated 10-fold by ultrafiltration through an Amicon DC-2 concentrator prior to ammonium sulfate fractionation.

Ammonium sulfate fractionation of the steroid-binding protein. Streptomycin sulfate was added to the

Table 2. Ammonium sulfate fractionation of periplasmic androgen-binding protein of *P. testosteroni*

Step	Binding activity (pmol steroid bound)	Recovery (%)	Specific activity (pmol/mg protein)
Lysozyme–EDTA supernatant	17.560		1.88
Ammonium sulfate (50–80%)	5.170	29.5	9.25

Note 1. Ammonium sulfate fractionation was performed on lysozyme–EDTA supernatant obtained from experiments shown in Table 1.

Note 2. Binding protein was dialyzed against 5 mM EDTA, 0.5 mM dithiothreitol, 2 nM [1.2-^{3}H]-androstenedione (2.3×10^7 c.p.m./nmol) and 50 mM Tris–HCl, pH 9.0, at 4°C for 20 h.

Table 3. Enzyme activities of ammonium sulfate fractions

Enzyme activity	Ammonium sulfate fractions % of activity present in Lysozyme–EDTA supernatant (0–50%)	(50–80%)
3β- and 17β-Hydroxysteroid dehydrogenase	23.8	0.2
3α-Hydroxysteroid dehydrogenase	56.0	0
5-ene-3-Ketosteroid isomerase	55.9	2.2
1-ene-Dehydrogenase	45.4	7.5
4-ene-5α-Dehydrogenase	0	0
4-ene-5β-Dehydrogenase	0	0

Note: The 3α-hydroxysteroid dehydrogenase and 3β- and 17β-hydroxysteroid dehydrogenase activities were measured as pmols of NAD^+ reduced per min per mg of enzyme protein. All other enzyme activities were measured as pmol of steroid metabolized per min per mg of enzyme protein. The results are represented here as % of activity present in the lysozyme–EDTA supernatant.

Table 4. Ammonium sulfate fractionation of a periplasmic estradiol-binding activity from *P. testosteroni*

Experiment	Estradiol-binding Activity Periplasmic fluid	Ammonium sulfate fraction (0–50%)	(50–80%)
	(pmol steroid bound)		
1	1080	831	114
2	534	437	60
3	1432	1624	111

Note 1. In Experiment 1, periplasmic fluid was prepared by osmotic shock, whereas in the other two experiments the periplasmic fluid was prepared by lysozyme–EDTA treatment. The ammonium sulfate fractionation was performed on periplasmic fluid obtained from experiments shown in Table 1.

Note 2. Binding protein was dialyzed against 1.7 nM [6,7-^{3}H]-estradiol-17β (3.3×10^7 c.p.m./nmol).

periplasmic fluid to a final concentration of 1% (w/v) and the precipitated DNA removed by centrifugation. To the supernatant, ammonium sulfate was added with stirring at 4°C to achieve the saturation desired. The precipitate was harvested by centrifugation at 8000 *g* for 20 min at 4°C. The precipitated protein was dissolved in a solution containing 50 mM Tris–HCl, pH 9.0, 5 mM EDTA, and 1.0 mM dithiothreitol. Protein concentrations were determined by the method of Lowry[10].

Binding assay. Steroid-binding activity of periplasmic protein was analyzed by equilibrium dialysis technique as described [7]. Usually, two concentrations of periplasmic protein, in a total volume of 1.0 ml, were dialyzed against 15 ml of a solution containing 50 mM Tris–HCl, 5 mM EDTA, 0.5 or 1 mM dithiothreitol, and radioactive steroid. After 20 h the

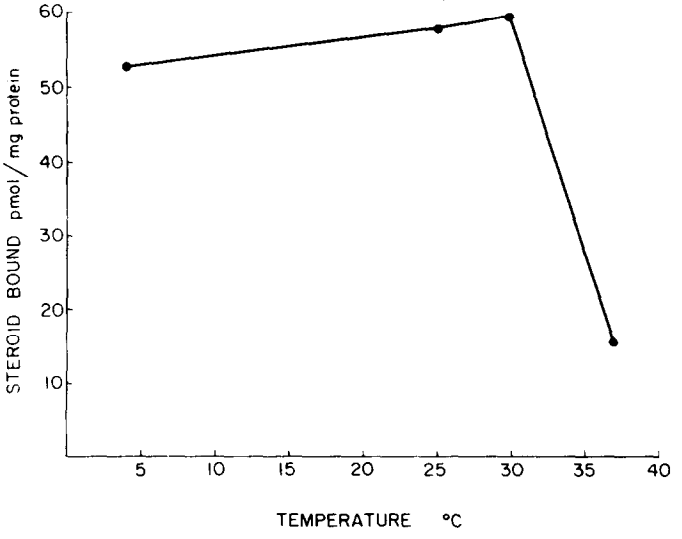

Fig. 1. Effect of temperature on steroid binding activity. Two concentrations of periplasmic protein, in a total volume of 1.0 ml, were dialyzed against 15 ml of a solution containing 50 mM Tris–HCl, pH 7.0 and 128 nM [7-^{3}H]-testosterone (3.3×10^6 c.p.m./nmol). EDTA and dithiothreitol were omitted from the reaction mixture. The incubation temperatures varied from 4 to 37°C.

Table 5. Effect of preincubation at 37°C on steroid binding activity of partially purified periplasmic protein

Time of Preincubation at 37°C (min)	Assay temperature (°C)	Testosterone bound (pmol/mg protein)
0	30°	68.56
30	30°	70.26
90	30°	72.23
150	30°	86.95
0	37°	10.13

Note: Two concentrations of periplasmic protein, in a total volume of 1.0 ml were dialyzed against 15 ml of a solution containing 50 mM Tris–HCl, pH 7.0, and 128 nM [7-^{3}H]-testosterone (3.3 × 10^6 c.p.m./nmol). The assay mixture was incubated at 37°C for periods up to 150 min, and binding was assayed at 30°C for 20 h. One set was incubated at 37°C for 20 h.

radioactivity inside and outside the dialysis bag was determined in a liquid scintillation spectrometer.

Enzyme assays. The assays for 3α- and 3β- and 17β-hydroxysteroid dehydrogenases, 5-ene-3-ketosteroid isomerase, 1-ene-dehydrogenase, 4-ene-5α-dehydrogenase and 4-ene-5β-dehydrogenase were performed as previously described [8, 9, 11–13].

RESULTS

Release of periplasmic binding protein by lysozyme–EDTA treatment

We have previously reported that steroid-binding proteins and steroid transforming enzymes, induced by growth of *P. testosteroni* on various C19 and C21 steroids, can be released into the medium by exposure of cells to osmotic shock [7, 9]. Alternate methods for releasing periplasmic protein were examined since osmotic shock techniques occasionally released degradative enzymes (1-ene-dehydrogenase) [9] which made subsequent analysis of binding difficult due to loss of label from the [1,2-^{3}H]-labelled steroids. Secondly, since the amount of binding activity released by osmotic shock was variable from preparation to preparation, an alternate technique which would guarantee reproducible yields of binding proteins was desired.

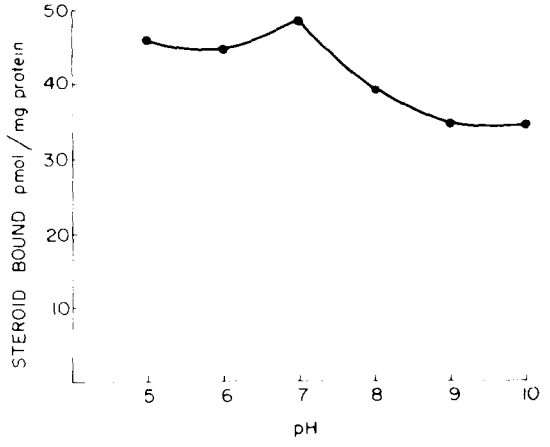

Fig. 2. Effect of pH on steroid binding activity. The experiment was performed as described in Fig. 1 except that the pH of the dialysis medium varied from 5 to 10. The binding assay was performed at 30°C.

Induced cells were initially exposed to osmotic shock treatment and subsequently subjected to lysozyme–EDTA treatment and the periplasmic protein thus obtained was examined for steroid-binding activity. It is evident that the amount of binding activity released by osmotic shock was variable from preparation to preparation (Table 1). Treatment of osmotically shocked cells with lysozyme–EDTA resulted in a further release of binding activity which was usually greater in amount than that released by osmotic shock. Treatment of induced cells with lysozyme–EDTA without prior osmotic shock also released steroid-binding activity (Exp. 7, Table 1). There was some variability in the total amount of binding activity released from experiment to experiment but because of the greater yield of binding activity when cells were treated with lysozyme–EDTA, this method was used for subsequent preparations, omitting the initial exposure to osmotic shock.

Partial purification of the binding protein

Before studying the requirements for steroid binding, a partial purification of the binding protein was attempted. The lysozyme–EDTA supernatant was

Table 6. Effect of EDTA and dithiothreitol on steroid binding

Assay conditions	Steroid bound (pmol/mg protein)
Complete reaction mixture	57.9
Minus EDTA	51.6
Minus dithiothreitol	52.9
Minus EDTA and dithiothreitol	58.1

Note: Binding was assayed at 30°C as described in the text. In the complete reaction, binding protein was dialyzed against 50 mM Tris–HCl, pH 7.0, 5 mM EDTA, 1 mM dithiothreitol, and 128 nM [7-^{3}H]-testosterone (3.3 × 10^6 c.p.m./nmol).

Table 7. Effect of ions on steroid binding

Additions	Concentration (mM)	Steroid bound (pmol/mg protein)
None		52.2
$MnCl_2$	2.5	53.5
NaCl	2.5	61.7
$CaCl_2$	2.5	55.6
KCl	2.5	50.4
$CuSO_4$	2.5	60.1
$MgSO_4$	2.5	44.0
$ZnSO_4$	0.33	15.0
	2.5	3.6
$FeSO_4$	2.5	11.7
$FeCl_3$	2.5	13.5

Note. The ions were added to the dialysis medium without prior incubation with the binding protein. EDTA and dithiothreitol were omitted from the dialysis medium. The dialysis medium contained 50 mM Tris–HCl, pH 7.0, and 128 nM [7-^{3}H]-testosterone (3.3×10^6 c.p.m./nmol). The binding assay was performed at 30°C.

fractionated by ammonium sulfate. The 50–80% saturated fraction contained approximately 30% of the recovered androgen-binding activity and yielded a 5-fold purification (Table 2). The 0–50% fraction contained undetectable amounts of androgen-binding activity.

Steroid transforming enzymes present in the periplasmic space are also released by osmotic shock [9]. Lysozyme–EDTA treatment also released these enzymes but on ammonium sulfate fractionation the bulk of the enzyme activities appeared in the 0–50% saturated fraction (Table 3). The estradiol-binding protein [9] also separated predominantly into the 0–50% saturated fraction (Table 4).

Requirements for steroid binding by periplasmic protein

The effect of temperature on steroid binding activity of induced bacterial cells was previously reported [8]. Binding activity was inhibited when the assay was performed at 37 or 45°C, although the inhibition at 37°C appeared to be reversible [8]. Similarly, when binding activity of partially purified periplasmic protein was assayed at different temperatures the optimum temperature for binding was 30°C, with marked inhibition at 37°C (Fig. 1). As with the intact organism, the inhibition at 37°C was reversible (Table 5). Binding protein incubated at 37°C for up to 150 min retained binding activity when subsequently restored to the assay temperature of 30°C.

The effect of pH on binding was reexamined at 30°C since our previous studies had been performed at 4°C [7]. The optimum pH for binding appeared to be 7.0, although binding was evident between the pH ranges of 5 to 10 (Fig. 2). Our previous studies at 4°C failed to demonstrate an effect of pH on steroid binding activity.

We have usually used EDTA and dithiothreitol in our binding assays, although omitting them from the reaction mixture did not significantly affect the binding activity (Table 6). We have also examined requirements for various ions but no specific requirement could be determined (Table 7). There was, however, a significant inhibition of binding when $ZnSO_4$, $FeSO_4$ or $FeCl_3$ was present in the assay mixture.

Substrate specificity

The specificity of the binding was examined on the 50–80% ammonium sulfate fraction since this step removed most of the steroid transforming enzymes. The specificity of binding, however, did not differ at this stage of purification from that observed with the osmotic shock protein [9] (Table 8). There was some binding observed with deoxycorticosterone, corticosterone and cortisol which was not previously observed.

Kinetics of binding

When partially purified periplasmic steroid-binding protein was assayed by equilibrium dialysis at 30°C with increasing concentrations of testosterone, the kinetics of binding was represented by a hyperbolic curve (Fig. 3), indicating a simple equilibrium binding process involving independent binding sites. The data plotted according to the method of Hill[14] yielded a Hill coefficient of $n = 1.1$, indicating noncooperativity of binding sites (Fig. 4).

DISCUSSION

Periplasmic binding proteins have been isolated from a number of gram-negative organisms. It is generally believed that they are involved in the active transport of substrates which they bind because release or removal of the periplasmic binding proteins leads to reduction in transport and because specificity of binding generally reflects specificity of transport [1, 15–17]. However, reconstitution studies to find direct evidence for a role of binding proteins have been generally discouraging and the exact role of the periplasmic binding proteins in transport and other

Table 8. Binding of C18, C19 and C21 steroids by a 50–80% ammonium sulfate fraction of periplasmic protein

Steroid	Binding activity (pmol/mg protein)
Testosterone	76.8
Androstenedione	75.7
Androsterone	121.4
Dehydroepiandrosterone	138.5
Androstanedione	79.5
Dihydrotestosterone	40.3
Epitestosterone	36.3
17α-Hydroxyprogesterone	192.1
Progesterone	377.4
Deoxycorticosterone	22.7
Corticosterone	23.6
Cortisol	14.9
Estradiol-17β	17.5

Note. The dialysis medium contained 50 mM Tris–HCl, pH 7.0, and 100 nM radioactive steroids and the binding assay was performed at 30°C.

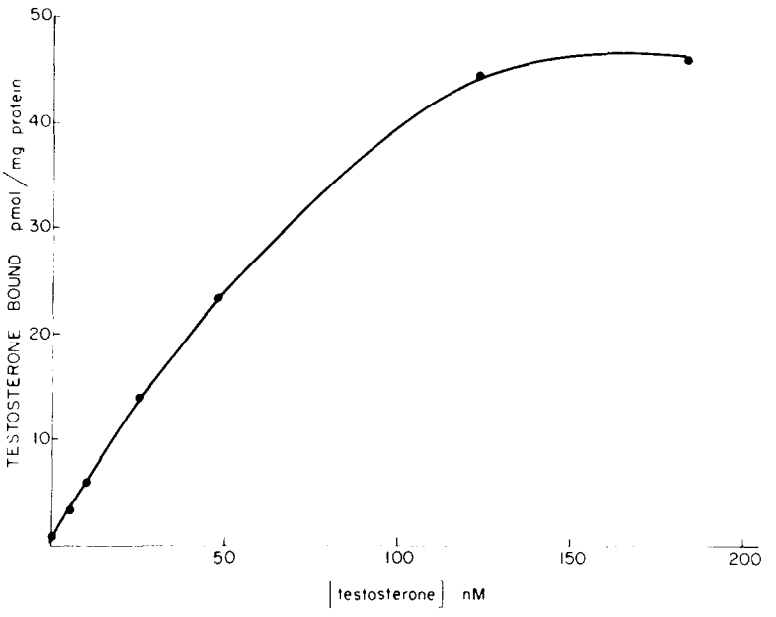

Fig. 3. Effect of testosterone concentration on steroid binding activity. The experiment was performed as described in Fig. 1 except that testosterone concentration varied from 1 to 184 nM and the incubation temperature was 30°C.

membrane-associated phenomena have not been elucidated. In a recent review [17], Oxender concludes that the binding proteins appear to increase the affinity of the transport system for the solute by interacting with a membrane component. He suggests that the solute-binding protein complex may be the actual substrate for the membrane transport system.

A cooperative behaviour on binding of substrates with binding proteins also provides evidence for functional significance. Several reports have suggested the presence of cooperative behaviour in estradiol binding to proteins in cell-free extracts from uterus at 0°C [18, 19], but numerous other studies have been unable to confirm this finding [20–23].

In our studies of the periplasmic steroid-binding protein of *P. testosteroni*, some initial studies of the kinetics of binding suggested a cooperative interaction between ligand and binding protein [24]. More detailed studies, however, of binding protein partially purified by ammonium sulfate fractionation have

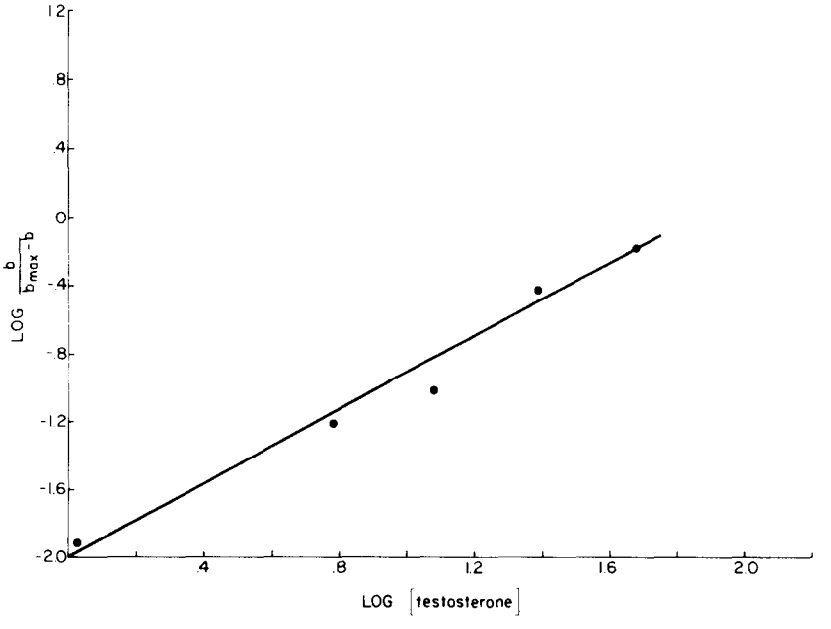

Fig. 4. Hill plots of testosterone binding by partially purified steroid-binding protein. The plots were derived from data shown in Fig. 3.

failed to demonstrate cooperative binding behaviour. The results are consistent with a simple equilibrium binding process with independent binding sites.

The studies reported here were performed on periplasmic binding proteins partially purified by ammonium sulfate fractionation. Although the bulk of the periplasmic steroid transforming enzymes and estradiol binding proteins were removed during this purification step, no change in binding properties or substrate specificity was observed. Further purification procedures are currently being examined in our laboratory in order to characterize further the periplasmic steroid-binding protein. It would be important to test binding specificity, specific requirements for binding with each steroid bound, and the kinetics of binding on a more purified preparation once this becomes available.

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