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BINDING OF STEROIDS TO P. TESTOSTERONI Δ^5-3-KETOSTEROID ISOMERASE: MEASUREMENT OF THE NUMBER OF BINDING SITES BY EQUILIBRIUM DIALYSIS

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SUMMARY

The binding of progesterone, 17β-estradiol and 19-nortestosterone acetate to the Δ^5-3-ketosteroid isomerase from Pseudomonas testosteroni has been investigated by the technique of equilibrium dialysis. Under the conditions used, all three steroids formed 2:1 complexes with each molecule of enzyme dimer (M.W. = 26,788). No evidence of any cooperative binding phenomena was obtained. The dissociation constants of the enzyme steroid complexes at 25°C were: progesterone, 2.2 μM; estradiol, 2.5 μM; 19-nortestosterone acetate, 9.2 μM.

INTRODUCTION

Recently we described the chemical characterization of the 19-nortestosterone acetate dependent photoinactivation of the Δ^5-3-ketosteroid isomerase (1). In this study and that of Martyr and Benisek (2) it was found that the 19-nortestosterone acetate sensitized photoinactivation of the enzyme by light of $\lambda > 300$ nm was due to the conversion of residue 38 of the polypeptide chain (aspartic acid in the enzyme preparations used) to alanine. The extent of inactivation correlated with the modification of one residue of aspartic acid per polypeptide chain. Since the enzyme is a dimer of identical polypeptides under most solution conditions including those of the photoinactivation reaction (3-5), our experiments were consistent with the presumed existence of two identical 19-nortestosterone acetate binding sites per molecule of enzyme dimer. Subsequent to

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these reports Vincent et al. (6) published a study of the binding of three steroids to P. testosteroni Δ^5-3-ketosteroid isomerase by equilibrium dialysis and fluorescence quenching titration. These workers found that, in their hands, only one molecule of steroid was bound by each molecule of dimeric enzyme. The apparent discrepancy between the photoinactivation work and the equilibrium binding studies prompted us to independently reinvestigate the binding of steroids to the isomerase using a similar enzyme preparation as was employed in the photoinactivation experiments. The method of equilibrium dialysis was chosen for this purpose since it "senses" all binding sites independently of any special spectroscopic effects induced by steroid binding. Using this classical technique (7) we have measured the number of steroid binding sites per polypeptide chain, n, and the apparent dissociation constant, K_d, for three steroids known to be competitive inhibitors of the enzyme. The steroids employed were progesterone, estradiol and 19-nortestosterone acetate. The results we have obtained differ significantly from those of Vincent et al. (6) with regard to the number of steroid binding sites detected.

MATERIALS AND EXPERIMENTAL PROCEDURES

Δ^5-3-Ketosteroid isomerase was prepared by the procedures of Jarabak et al. (8) and Benson et al. (9) with the affinity column chromatography followed by pH 4-6 preparative isoelectric focusing followed by ampholyte removal on a column of Sephadex G-50. Enzyme so purified showed only one band either on SDS gels or on pH 4-6 IEF gels. The specific activity of the purified enzyme was 5.16×10^4 IU/mg.

6,7-[^{3}H]-17β-estradiol (44.0 Ci/mmol) and 1,2-[^{3}H]-progesterone (55.7 Ci/mmol) were from New England Nuclear and were radiochemically diluted with unlabeled steroid (from Steraloids) to give specific activities at usable levels (48.91 Ci/mol in the case of progesterone; 165.5 Ci/mol for estradiol). They were then purified by preparative thin layer chromatography on silica gel using cyclohexane:ethyl acetate (1:1 vol) as the chromatography solvent. Chemical and radiochemical purity were then checked by thin layer chromatography on E. Merck silica gel F254 using cyclohexane:ethyl acetate (1:1 vol) and chloroform:methanol (9:1 vol) for both steroids followed by autoradiography using Kodak No-Screen X-ray film. 4-[^{14}C]-nortestosterone acetate was obtained from New England Nuclear and checked for purity via the thin layer chromatographic and autoradiographic procedures mentioned previously. The specific activity of this preparation was 2.69 Ci/mol.

Dialysis bags were prepared from Spectrapor 3 (M.W. cutoff = 3500) tubing by washing several times in 1 mM EDTA, 0.1% $NaHCO_3$ at 60°C followed by copious washing with distilled water at 50-60°.

Enzyme previously dialyzed against 34 mM potassium phosphate, 2.5 mM EDTA, pH 7.0 was diluted to an appropriate concentration (1 to 10 μM) with the same buffer. This solution was then made 1.66% in methanol and 1.0 ml aliquots were transferred to the dialysis bags. Each bag was then sealed by double knots and placed inside an 8 ml disposable glass scintillation counting vial (Brockway Glass Co.) which had previously received 3.0 mls of the above buffer plus 50 μl of the steroid in methanol. The vials were then stoppered, secured in a ten-hole fraction collecting rack (LKB 7005-2) and rotated at approximately 10 rpm at an angle of about 45° from the horizontal such that mixing was facilitated without liquid ever touching the polyethylene vial cap. Rotation was performed for 18-20 hours (24 hours for nortestosterone acetate) at either 25°C or 4°C. For all steroids the time to equilibrium was approximately 12 hours. In the nortestosterone acetate experiments, steroid was introduced on both sides of the bag at the beginning of the dialysis. Upon completion of the equilibration period, the contents of the vials and bags were transferred to small glass test tubes, from which duplicate or triplicate aliquots (0.1 to 0.3 ml) were withdrawn for scintillation counting. Samples were counted in 6 mls of Amersham PCS scintillation cocktail in a Beckman LS-255 counter. Assays of all solutions for enzymatic activity after the experiment confirmed that the enzyme activity was stable over the time span of the dialysis and that no leakage of enzyme from the bag had occurred.

The concentration of unbound steroid, F, was calculated from the radioactivity of the solution outside the bags. The concentration of bound steroid, B, was calculated from the difference in the radioactivity of the solution inside the bag and that outside the bag.

The protein concentration of the stock enzyme solution added to the dialysis bags was determined by measurement of the absorbance at 280 nm. An absorbancy coefficient of 0.336 $(mg/ml)^{-1}$ was assumed (3). All enzyme concentrations, E_T, are expressed as the molar concentration of monomer assuming a monomer molecular weight of 13,394 (calculated from the amino acid sequence of Benson et al. (12)).

The dialysis experiments were analyzed graphically by the method of Scatchard (13). The plots of bound steroid/free steroid (B/F) versus bound steroid (B) were fitted to straight lines by the method of least squares using a programable Hewlett Packard Model 9825A desk top computer equipped with Hewlett Packard X-Y plotter, Model 9862A. From the straight line functions the values of n, the number of binding sites per monomer, and K_d the dissociation constant of the steroid-monomer complex were determined from the slope and intercepts of the Scatchard plots.

RESULTS AND DISCUSSION

The results of the binding experiments are shown in Figures 1-3 in the form of the computer generated Scatchard plots. For the three steroids examined the data could be fit by linear functions. No evidence of curved or biphasic-linear functional behavior was apparent in the data points. In Table I the values of n and K_d obtained from each of the Scatchard plots are presented. All three steroids formed 1:1 complexes with each

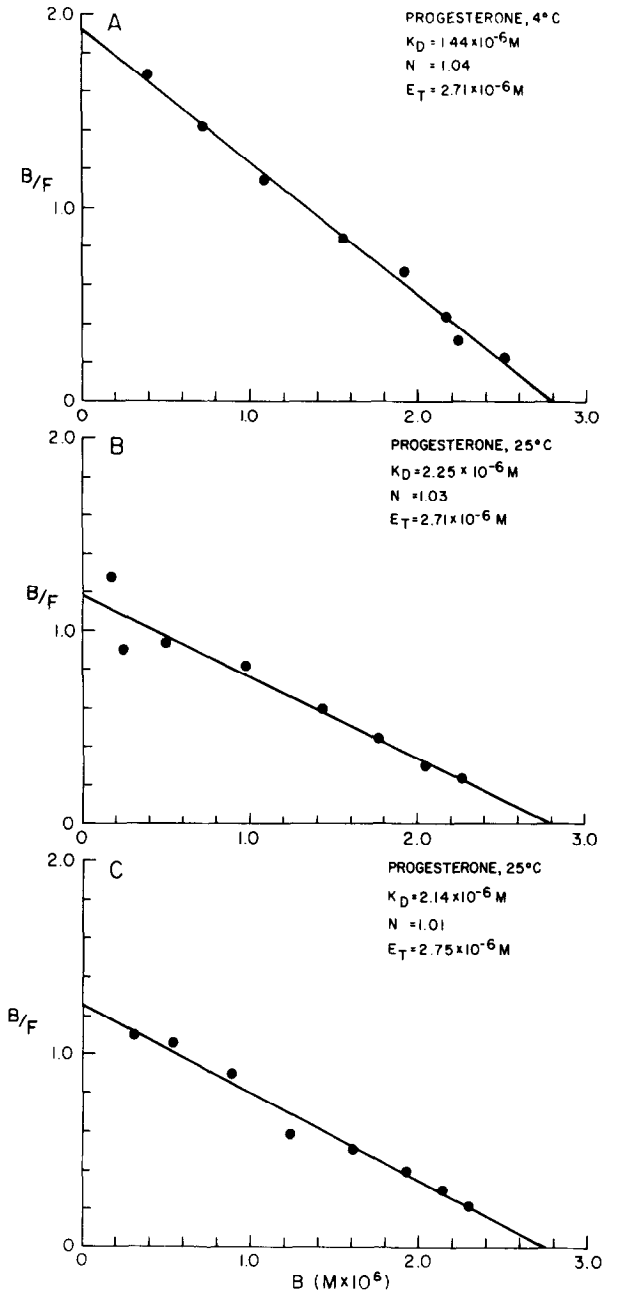

Figure 1. Scatchard Plots of the Binding of Progesterone to Isomerase. A, at 4°C, B and C are duplicate experiments at 25°C. Other conditions are described in Materials and Experimental Procedures.

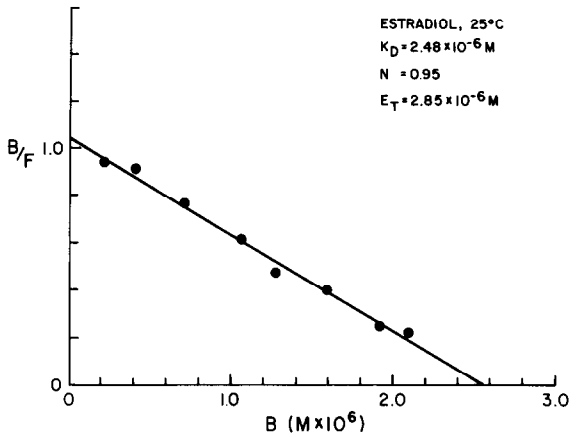

Figure 2. Scatchard Plot of the Binding of 17β-Estradiol to Isomerase at 25°C. Other conditions are described in Materials and Experimental Procedures.

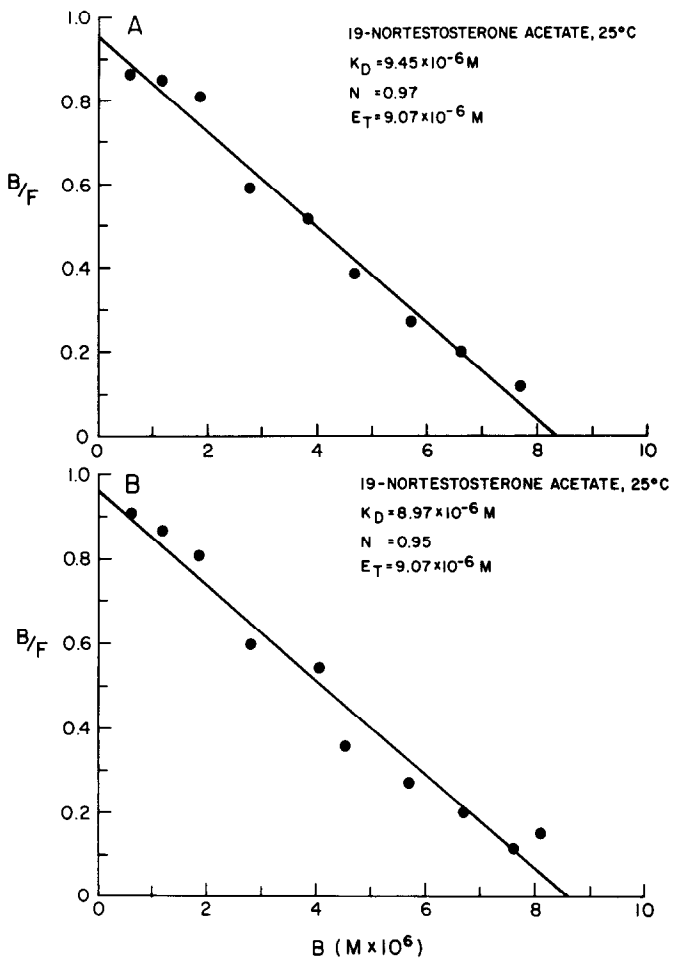

Figure 3. Scatchard Plots of the Binding of 19-Nortestosterone Acetate to Isomerase at 25°C. Other conditions are described in Materials and Experimental Procedures. A and B are duplicate experiments.

TABLE I

PARAMETERS FOR THE BINDING OF VARIOUS STEROIDS TO P. TESTOSTERONI

Steroid	Figure	n[a]	K_d (μM)	E_T[b] (μM)	T (°C)
progesterone	1A	1.04	1.44	2.71	4
progesterone	1B	1.03	2.25	2.71	25
progesterone	1C	1.01	2.14	2.75	25
17β-estradiol	2	0.95	2.48	2.85	25
19-nortestosterone acetate	3A	0.97	9.45	9.07	25
19-nortestosterone acetate	3B	0.95	8.97	9.07	25

[a] n, number of binding sites per monomer (M.W. 13,394)

[b] E_T, concentration of isomerase monomer

enzyme subunit, i.e., each molecule of dimeric enzyme bound 2 molecules of steroid in a site-site independent fashion, albeit with different values of K_d for each steroid.

These data are fully consistent with the results of site-specific chemical modifications by photoactivated 19-nortestosterone acetate (1,2) and by the "suicide" 5,10-seco-steroid substrates employed by Penning et al. (10). In the photoinactivation studies, the loss of enzyme activity was correlated with the photodecarboxylation of one residue of aspartic acid in each subunit. Penning et al. (10) found that each isomerase subunit incorporated one molecule of [7-^{3}H]-5,10-secoestr-5-yne-3,10,17-trione during inactivation by this reagent. Thus, both chemical modification studies suggest the presence of two steroid binding sites per enzyme dimer. Our results are also in agreement with those of Wang et al. (11) who determined the number of steroid binding sites by spectrophotometric and spectrofluorometric titrations with 19-nortestosterone. These workers found

that 40,800 grams of isomerase bound 2.8-3.1 moles of 19-nortestosterone. Since the molecular weight of the isomerase dimer is now known to be near 27,000 (3-5), the results of Wang et al. (11) are equivalent to a steroid to dimer binding stoichiometry of 1.85-2.05. This conclusion is now fully supported by the present results, at least for the three steroids investigated, one of which, 19-nortestosterone acetate, was the steroid used in the active site-directed photoinactivation studies.

Our results are in conflict with those of Vincent et al. (6) who detected in their enzyme preparations only one steroid binding site per enzyme dimer. These workers measured the binding of 19-nortestosterone, 17β-estradiol and equilenin under conditions very similar to those employed by us. It should be noted that both groups have examined 17β-estradiol binding, obtaining these markedly different results for the binding stoichiometry. Thus the discrepancy cannot be due to a choice of different steroids for study. At the present time we can offer no convincing explanation for the difference in binding stoichiometry determined by us and Vincent et al. (6). Further work is necessary in order to resolve the question.

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