'HALF-OF-THE-SITES' REACTIVITY OF STEROID ISOMERASE

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1. Introduction

Pseudomonas testosteroni $\Delta_{5\rightarrow4}$ -3-oxosteroid isomerase (EC: 5.3.3.1) [1] is a dimer of approx. 27 000 daltons [2], composed of two polypeptide chains of of identical molecular weight [2–4] and primary sequence [5]. In this work, the binding of three competitive inhibitors to isomerase has been studied by different techniques. The results obtained by equilibrium dialysis and by measurements of the ultraviolet fluorescence of the protein have been compared. The dissociation constant for each of the three ligands as measured by these two techniques and by kinetic studies are in very good agreement. Both approaches agree in assigning only a single binding site per isomerase dimer; a second weaker binding site, if any exists, could not be evidenced under the present experimental conditions. Such an apparent reactivity of only half of the enzyme sites has been described with different intracellular oligomeric enzymes involved in metabolic pathways (e.g. alkaline phosphatase from *E. coli*), and the functional evolutionary interest of the interdependence of the subunits in the catalytic process has been discussed [6,7]. As far as we know, this 'half-of-the-sites' reactivity is the first one observed with an enzyme involved in steroid hormone metabolism. This finding of a single binding site for steroids which are either substrate analogs or products, opens the question of the functional unequality of the two potential binding sites in the isomerase dimer, and consequently of a possible regulatory mechanism applicable to such an enzyme under physiological conditions.

2. Experimental procedure

2.1. *Isomerase*

Isomerase was purified and crystallized as usually [8–10]. Its homogeneity was checked by polyacrylamide gel electrophoresis and by its ultraviolet characteristic spectrum. Pure isomerase has a specific activity of about 55 000 units/mg, using the new $E^{0.1\%}_{1\ cm}$ value at 280 nm of 0.328 ± 0.004 [2]. One unit of enzyme isomerizes 1 μmol of Δ^5-androstene-3,17-dione per min under standard conditions [11].

2.2. *Steroids and measurements of radioactivity*

Labelled steroids: [6,7-^{3}H]estradiol (spec. act.: 43 Ci/mmol), was purchased from N.E.N. (Boston, Mass.), [4-^{14}C]19-nortestosterone (spec. act.: 40 Ci/mmol) was a C.E.A. (Saclay, France) product. Purity was assessed by thin-layer chromatography.

Radioactivity was measured with a Packard liquid scintillation counter in 10 ml Bray's solution (efficiency 16–20%), or in 10 ml of 4 g Omnifluor (N.E.N.)/liter toluene (efficiency 40%).

Unlabelled steroids were given by Roussel-Uclaf and used without any further purification.

2.3. *Chemicals*

The potassium phosphate buffer was made with Merck products. Methanol and ethanol were from Prolabo ('ultrapur') and dioxan was from Merck (Uvasol for spectroscopy).

2.4. *Equilibrium dialysis*

Dialysis tubing (Visking tubing 8/100 ft, Union Carbide Corp.) was successively boiled with sodium

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carbonate, then slowly cooled with EDTA solution and washed extensively with distilled water. Dialyses were performed at 4°C. One ml aliquots of isomerase solution in 0.03 M potassium phosphate buffer, pH 7.0, containing 1% v/v of methanol were dialyzed with magnetic stirring against 15 ml of the same medium, radioactive 19-nortestosterone or estradiol being introduced outside the tubing. After 20 hours (16 h is the time necessary to reach equilibrium), the radioactivity inside and outside the tubing was measured and steroid concentrations were calculated. The unbound steroid concentration (U) is equal to the steroid concentration outside the tubing; the bound steroid concentration (B) is obtained from the difference between the total steroid concentration (T) inside the tubing and (U). The equilibrium dissociation constant (K_D) and the concentration of binding sites (N) were determined according to Scatchard [12].

2.5. *Fluorescence titration by two competitive inhibitors*

The titration was performed with a Fica 55 recording differential spectrofluorimeter which gives absolute fluorescence spectra. Fluorescence intensities were expressed in arbitrary units and no correction for self absorption of the incident light was necessary since absorbancy at the excitation wavelength was $\leqslant 0.09$.

The fluorescence emission spectrum of isomerase, a protein devoided of tryptophan residues, has a maximum at 305 nm (maximum excitation wavelength 275 nm). The tyrosine fluorescence intensity at 305 nm of an isomerase solution is quenched by the stepwise addition of steroid ligands which are competitive inhibitors: 19-nortestosterone (Δ^4-androsten-3-one-17β-ol) and equilenin ($\Delta^{1,3,5,6,8}$-estra-penten-3,17-diol); since these steroids do not fluoresce at 305 nm when excited at 275 nm, it is possible to follow the isomerase–steroid interaction by a selective fluorescence quenching technique [13].

Isomerase titrations have been performed with silica cells containing 1.5 ml of an isomerase solution in 0.03 M potassium phosphate buffer, pH 7 at 26°C. The isomerase concentration range varied from 1.2 μM to 7.3 μM, where linearity between fluorescence and protein concentration is observed. After each addition of steroid dissolved in dioxan or methanol, the fluorescence intensity at 305 nm is measured when exciting the protein at 275 nm. Corrections for protein dilution were made from blank titrations with methanol or dioxan.

From the experimental data, the number of binding sites (N) and the dissociation constant (K_D) were determined according to graphical representations derived from the law of mass action, namely the Scatchard representation [12] and plots based on the equation $T/E_L = N + K_D/E_F$ where T is the final ligand concentration in the assay medium, E_L and E_F the concentration of liganded and free protein respectively. These two representations assume for isomerase one single molecular species, N independent binding sites equivalent with respect to affinity, and interaction of the ligand at each site occurring with the same fluorescence quenching.

3. Results and discussion

3.1. *Dialysis equilibrium studies*

Isomerase (1 to 4 μM) was exposed to different concentrations of radioactive 19-nortestosterone or estradiol which are competitive inhibitors of the isomerization reaction. The enzyme is not denatured during the time necessary for dialysis experiments, as checked by the measure of the enzymatic activities before and after dialysis. Fig.1 (curves a and b) shows the Scatchard plots (obtained in the presence of 1% v/v of methanol), giving for 19-nortestosterone a K_I

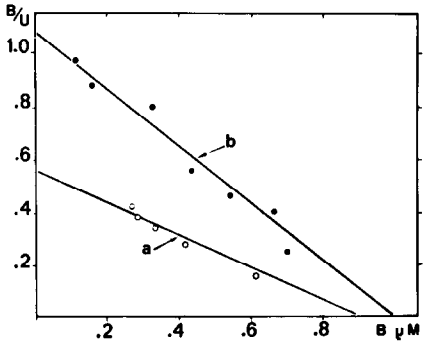

Fig.1. Scatchard plots of the binding of 19-nortestosterone (curve a) and estradiol (curve b) to isomerase 1.0 μM, as obtained by equilibrium dialysis experiments. Dialyses were performed in 0.03 M potassium phosphate buffer, pH 7.0, in 1.6% v/v of ethanol at 4°C. Results are K_I = 1.7 μM and N = 0.98 for 19-nortestosterone, and K_I = 0.85 μM and N = 1.08 for equilenin.

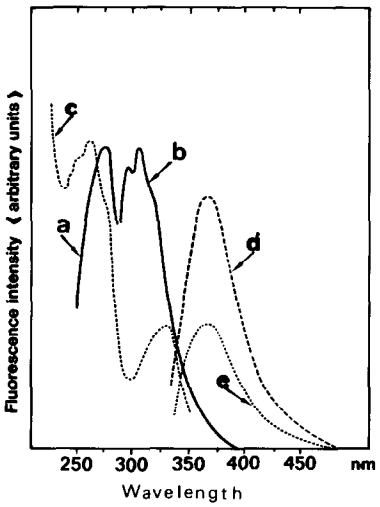

Fig.2. Excitation and emission spectra of isomerase (3.8 μM) and equilenin (5.4 μM) in 0.03 M potassium phosphate buffer, pH 7.0. Curves a and b are the excitation and emission spectra of isomerase. Curve c is the excitation spectrum of equilenin, d and e are emission spectra of equilenin excited at 275 nm and 335 nm, respectively.

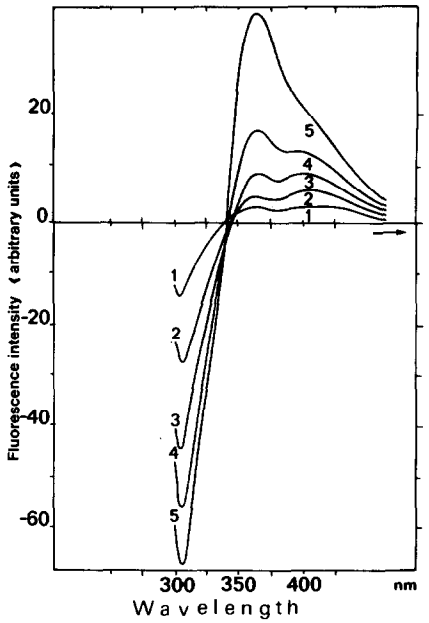

Fig.3. Fluorimetric difference spectra of isomerase and isomerase with increasing equilenin concentrations. The reference cell contains isomerase (7.1 μM) in 0.03 M potassium phosphate buffer, pH 7.0. The titration cell contains the same isomerase concentration (7.1 μM) in the same medium, with successively different equilenin concentrations : 1.80 μM, 3.58 μM, 5.35 μM, 7.1 μM and 10.6 μM.

value of 1.7 μM and a N value of 0.98 and for estradiol a K_I value of 0.85 μM and a N of 1.08. These results with two different competitive inhibitors therefore suggest one binding site per isomerase dimer.

3.2. *Fluorescence studies*

Fig.2, curves a and b, depicts the fluorescence excitation and emission spectra of an isomerase solution in 0.03 M potassium phosphate buffer, pH 7. The emission spectrum of isomerase is characterized by two neighbour peaks centered at 298 nm and 305 nm.

Fig.2, curve c, shows the excitation spectrum of equilenin in the same medium but containing 2% v/v of dioxan; it displays two peaks located at 265 nm and 335 nm. Curves d and e show the emission spectra of free equilenin when excited at 275 nm and 335 nm respectively. The same maximum is observed and the fluorescence intensity of the trailing part of the emission spectrum at 305 nm is negligible.

Fig.3 represents the differences emission spectra of a reference isomerase solution (7.1 μM), in 0.03 M potassium phosphate buffer, pH 7, against the same solution of isomerase with increasing equilenin concentrations. Fluorescence modifications are observed both in the protein and equilenin region. In the equilenin fluorescence region, two emission peaks are observed at 365 nm and 405 nm at low equilenin concentrations. The last one (405 nm) can be related to the complex formation. At higher equilenin concentrations, the percentage of free equilenin with respect to the complex increases and the emission peak at 365 nm previously described overlaps the peak at 405 nm. In the protein fluorescence region, the decrease of fluorescence intensity at 305 nm (fig.3) corresponds to the quenching of the emission of isomerase when interacting with equilenin.

Fig.4 depicts the dependence of fluorescence quenching of isomerase (3.8 μM) as a function of the final ligand concentration.

The saturation curves with 19-nortestosterone (fig.4, curve a) or equilenin (curve b) may be analyzed to yield the binding constant i.e. N and K_D [13]. For this purpose, it is necessary to relate the observed

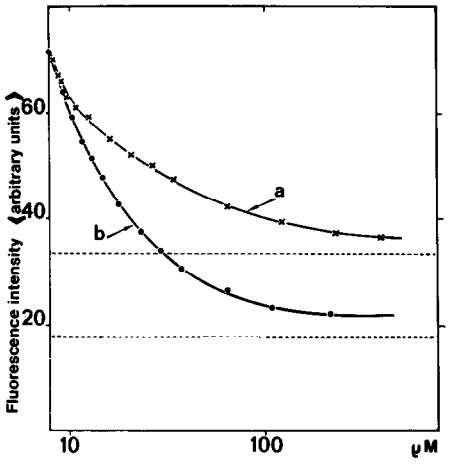

Fig.4. Fluorescence saturation curves of isomerase (3.8 μM) with 19-nortestosterone (a) and equilenin (b). Experiments were performed in 0.03 M potassium phosphate buffer, pH 7.0 and 26°C, at emission wavelength 305 nm (excitation wavelength 275 nm). Q_m for 19-nortestosterone is equal to 10 arbitrary fluorescence units and to 14.2 arbitrary fluorescence units for equilenin.

degree of quenching either to the number of moles of steroid bound per mole of isomerase dimer for Scatchard plots [12] or to the concentration of liganded

protein (E_L) for graphs based on $\frac{T}{E_L} = N + \frac{K_D}{E_F}$

The E_L calculation requires the determination of Q_m, which corresponds to the maximum fluorescence decrease of a μM isomerase solution occurring when isomerase is totally complexed by the steroid. Experimentally, Q_m is obtained from the difference between the fluorescence intensity I_o of a μM free isomerase solution (I_o = 20 fluorescence arbitrary units) and the residual fluorescence intensity I_r when this μM protein solution is saturated by the ligand ($Q_m = I_o - I_r$). This I_r value was extrapolated from experimental data obtained at ligand concentrations T equal or higher than 9 times the protein concentration. By plotting $1/I_f$ as a function of $1/T$ (I_f are the fluorescent intensities at different ligand concentrations), a straight line is obtained: its intercept on the ordinate axis gives I_r. Thus, to any quenched fluorescence intensity ($Q = I_o - I_f$) of the saturation curve at a ligand concentration (T), corresponds a concentration E_L equal to $\frac{Q}{Q_m}$ and E_F is equal to $E_T - E_L$ (E_T being the enzyme concentration). By plotting T/E_L as a function of $1/E_F$, a straight line was obtained, with a slope K_D and an intercept on the ordinate equal to N. The average Q_m values (expressed in arbitrary fluorescence units per μM isomerase solution) are 15 and 10 for 19-nortestosterone and equilenin respectively.

For Scatchard plots, the 'Q_o' value which corresponds to the quenching efficiency of a μM bound steroid solution, is experimentally determined from the plateau value obtained by plotting the quenched intensities divided by ligand concentration in μM as a function of protein concentration (using the lowest steroid concentrations giving a fluorescence quenching response) (fig.5). The Q_o values (15 for equilenin and 10 for 19-nortestosterone) were obtained for the highest possible protein concentrations 7 μM (since at higher concentration, there is no linearity between fluorescence intensity and protein concentration). Thus, the concentration of bound steroid (B) at any fluorescence quenching intensity (Q) of the saturation curve is given by the ratio Q/Q_o. The unbound steroid concentration (U) was obtained from the difference between the total ligand concentration (T) and (B). The slope of the plot (B)/(U) versus (B) gave $1/K_D$ and the intercept on abscissa NE_o, E_o being the protein molar concentration and N the number of binding sites per isomerase dimer.

Fig.6a represents the Scatchard plot and fig.6b the graph T/E_L versus $1/E_F$ obtained with 1.1 μM of

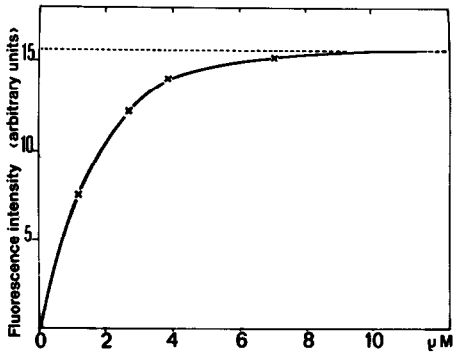

Fig.5. Determination of 'Q_o' value of equilenin. Plot of the quenched intensity per μM of ligand as a function of protein concentration.

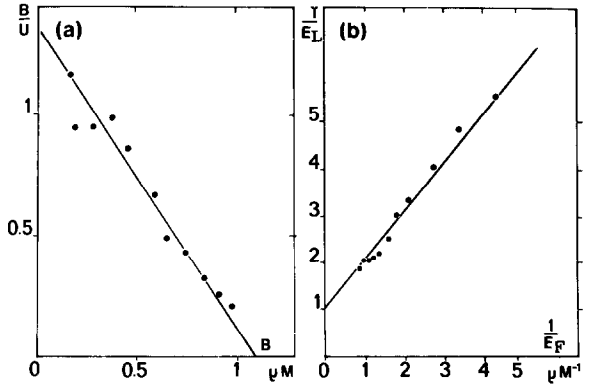

Fig.6. Spectrofluorimetric equilenin isomerase titration. Isomerase concentration was 1.1 μM. Excitation and emission wavelength were 275 nm and 307 nm, respectively.
(a) Scatchard plot. Data were plotted according to equation $B/U = NE_T/K_D - B/K_D$. (b) Data were plotted according to equation $T/E_L = K_I/E_F + N$. Experiments were performed in 0.03 M potassium phosphate buffer, pH 7.0 and at 26°C. The mean K_D and N values obtained are 1 μM and 1 binding site respectively.

isomerase (as titrated with equilenin). The two different plots gave a N value of 1.0 and a K_D of 1.0 μM.

The fluorescence results at different isomerase concentrations are summarized on table 1. As seen, the binding constants N and K_D are very similar whatever the protein concentration (1–7 μM) and the graphical representations. Again one binding site per dimer was obtained, and K_D were 1 μM and 2 μM for equilenin and 19-nortestosterone, respectively.

Finally the fluorimetric and dialysis equilibrium experiments using 19-nortestosterone and two estrogens which are competitive inhibitors of the isomerization reaction, indicate unambiguously the existence of a single binding site per isomerase dimer (table 2). The 2 methods give a very close K_D value for 19-nortestosterone. The low solubility of steroids in the aqueous medium do not allow to increase sufficiently the hormone concentration in order to demonstrate another, but weaker, binding site. On the basis of the identity of the 2 polypeptidic chains

Table 1
Fluorimetric determination of binding constants K_D and N of 19-nortestosterone and equilenin

Ligands / Isomerase concentrations (μM)	19-nortestosterone K_D (μM)	N	Equilenin K_D (μM)	N	Medium	% v/v
1.2	2.0	1.0[a]	1.1	0.9[a]	Dx	0 to 4
	2.3	1.0[b]	1.0	0.9[b]		
2.7			1.2	1.0[a]	Dx	0 to 4
			1.3	1.0[b]		
3.8	1.8	1.0[a]	1.0	1.0[a]	Dx	0 to 4
	1.5	1.0[b]	1.0	1.0[b]		
7.1			0.6	1.0[a]	MeOH	0 to 4
			1.1	1.0[b]		
7.3	1.5	0.8[a]			MeOH	0 to 4
	2.1	0.9[b]				

Dx: dioxan; MeOH: methanol.
[a] from Scatchard plot.
[b] from graph $\frac{T}{E_L} = N + \frac{K_D}{E_F}$.

Table 2
Binding parameters of 19-nortestosterone and equilenin as determined by different methods

Ligand	K_I	K_D (μM) E.D	F	N E.D	F
19-Nortestosterone	3.2	1.7	2.0	1	1
Equilenin	1.5	–	1	–	1

K_I: Inhibition constant as determined by kinetic study using Δ^5-androstene-3,17-dione as substrate in 0.03 M potassium phosphate buffer in 1.6% v/v of methanol.

K_D: dissociation constant as determined by Equilibrium dialysis (E.D) and fluorescence (F).

N: number of binding sites.

of isomerase, one may suggest that the 'half-of-the-sites' reactivity may imply a negative homotropic cooperativity in ligand binding.

Additional data will be reported [14] supporting the possibility of the flip-flop mechanism [5,6,15] for the isomerization process. They could shed some light on the apparently antinomic properties of the enzyme: Michaelian behaviour and 'half-of-the-sites' reactivity.

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