MOLECULAR WEIGHT DETERMINATION AND STRUCTURAL STUDIES OF *PSEUDOMONAS TESTOSTERONI* $\Delta_{5\rightarrow 4}$-3-OXOSTEROID ISOMERASE (EC 5.3.3.1)

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Received 3 August 1973

1. Introduction

The $\Delta_{5\rightarrow 4}$-3-oxosteroid isomerase (isomerase) was discovered by Wang and Talalay [1]. It was described as an oligomer of 40 800 mol. wt formed of three identical chains (see the most recent review [2]). Such results were obtained by using the 4.13 absorbance coefficient ($A^{1\%}_{1\,cm}$) in g/100 ml [3]. The amino acid composition [4] and the primary sequence of the subunit (mol. wt 13 600), were consistent with the postulated existence of three identical subunits. By spectrofluorimetric and spectrophotometric studies, Talalay et al. [5] have determined three binding sites for 19-nortestosterone and 17-β-hydroxy-equilenine.

However, our recent determination of the number of binding sites (to be published) does not fit the physical parameters given previously [2–4]. Therefore we have checked by different methods the $A^{1\%}_{1\,cm}$, the molecular weight of the oligomer and of the subunit in sodium dodecyl sulfate, the sedimentation coefficient ($s^0_{20,w}$) in the presence or in the absence of specific steroid ligands (competitive inhibitors), the Stokes radius (R_e), the frictional ratio (f/f_0), the partial specific volume ($\bar{v}$) and the isoelectric point (pI).

In the present communication are given all these physical parameters of isomerase which provide evidence that in the present experimental conditions, isomerase is a dimer, formed of two polypeptide chains of identical molecular weight.

2. Experimental procedure

Isomerase is purified and crystallized as usually [3, 6].

2.1. The $A^{1\%}_{1\,cm}$ at 277 nm and 280 nm is calculated from protein concentrations determined by different refractometry with a Perrin refractometer and absorbances measured on a 'Cary 15' spectrophotometer. The protein solutions are carefully dialysed against potassium phosphate buffer 0.03 M, pH 7 at 4°C. Three different isomerase concentrations correspond-

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ing to 243 500 units /ml (u/ml), 143 000 u/ml and 103 000 u/ml are used [1, 3].

2.2. *Molecular weight determination*

2.2.1. By light scattering [7–9].

Measurements are performed at angle values of 75°, 90°, 105°, 120° and 135° with a Fica 50 light-scattering apparatus at different protein concentrations (1.63 mg/ml), 2.13 mg/ml, 2.74 mg/ml and 3.60 mg/ml) determined with the new $A^{1\%}_{1cm}$ value.

2.2.2. By ultracentrifugation equilibrium

The molecular weight was determined by ultracentrifugation equilibrium according to the Yphantis technique [10, 11] on a Beckman model E ultracentrifuge. Isomerase concentration varies from 0.3 mg/ml to 0.6 mg/ml. The ultracentrifugations are carried out at 15°C at 40 000 rpm for 48 hr, with a double sector cell and interferential filter.

2.2.3. By gel filtration on Sephadex G-100

Columns (2.5 X 47 cm, 1.5 X 47 cm and 1.5 X 95 cm) are equilibrated at 10°C with potassium phosphate buffer 0.03 M, pH 7; 0.05–0.12 M KCl, the flow rate is about 8 ml/hr per cm^2 section.

Proteins used for calibration are: ribonuclease; Soybean trypsin inhibitor; chymotrypsinogen (Sigma Chemical Company); peroxidase (Worthington Biochemical Corporation); ovalbumin (Nutritional Biochemicals Corporation). Each sample layered on the top of columns contains a mixture of isomerase, blue dextran, potassium bichromate and different markers. Elution profiles are followed by measuring isomerase [1, 3] and peroxidase [12] activities; the other standard proteins are detected by their absorbances at 280 nm. Blue dextran is tested at 650 nm and potassium bichromate at 374 nm.

2.3. *Subunit molecular weight*

The subunit molecular weight is determined by polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate (SDS) according to Osborn et al. [13].

The standard proteins are: lysozyme (Miles Laboratories, Inc.); trypsin; chymotrypsin; pepsin (Sigma Chemical Company); ovalbumin and ribonuclease previously described.

2.4. *Sedimentation coefficient* $s^0_{20,w}$

Sedimentation velocity measurements of isomerase in the presence or in the absence of specific steroid ligands: 19-nortestosterone and estradiol (competitive inhibitors of isomerase) are performed in a linear sucrose gradient 5–20% in potassium phosphate buffer 0.03 M, pH 7, according to Martin and Ames [14] in a Spinco L_2B, with rotors SW 56 and SW 65 at 56 000 and 65 000 rpm at 4°C and 20°C for 20 hr. Bovine serum albumin (BSA, $s^0_{20,w} = 4.3 \times 10^{-13}$ sec) and peroxidase ($s^0_{20,w} = 3.55 \times 10^{-13}$ sec) are respectively used as external and internal markers. The BSA $s^0_{20,w}$ value is obtained from analytical ultracentrifugation experiments (Schlieren), and extrapolated at zero concentration. Isomerase and peroxidase are tested by their enzymatic activities and BSA by absorbance at 240 nm. In the experiments performed with competitive inhibitors the complexes 19-nortestosterone–isomerase and estradiol–isomerase are detected in the different fractions by the isomerase activity and by radioactivity of estradiol-6,7-^{3}H (N.E.N. specific activity = 43 Ci/mM) or 19-nortestosterone-4-^{14}C (C.E.A., specific activity = 45 mCi/mM). The radioactivity is measured with a Packard liquid scintillation counter in 10 ml Bray's solution, after addition of 0.5 ml of water to each fraction.

2.5. *The isoelectric point*

The isoelectric point (pI) is determined with an LKB 8101 column according to Svensson et al. [15, 16]. Ampholine Carrier ampholytes covering 7 pH units are obtained from LKB as a 40% solution. The ampholytes varied from 1% to 8% in a sucrose gradient from 0% to 46.6%. Twenty-four layering fractions of 4.6 ml are used. Electrofocusing is carried out for 20 hr at 10°C. In each fraction collected (2.8 ml), the isomerase activity and the pH are measured.

3. Results and discussion

3.1. *Absorbance coefficient* $A^{1\%}_{1cm}$

It has been shown that the refractive index difference (Δn) between a solution mixture containing a macromolecular component (i.e. a protein) and a solution without this component is directly proportional to the protein concentration (expressed in

Table 1
$A^{1\%}_{1cm}$ determination.

Activity of isomerase in units/ml	λ in nm	Absorbances (A)	Δx	k	$A^{1\%}_{1cm}$
243 500	280	1.30	223	$33 \cdot 10^{-7}$	3.28
	277	1.48			3.72
243 500	280	1.30	424	$17.5 \cdot 10^{-7}$	3.24
	277	1.48		$17.5 \cdot 10^{-7}$	3.68
149 500	280	0.895	284	$17.5 \cdot 10^{-7}$	3.33
	277	1.02		$17.5 \cdot 10^{-7}$	3.76
103 200	280	0.633	204	$17.5 \cdot 10^{-7}$	3.29
	277	0.72		$17.5 \cdot 10^{-7}$	3.74

mg/ml) and is independent of the state of polymerisation of the macromolecule [17].

$$\Delta n = k \cdot \Delta x = \frac{dn}{dc} \times C$$

dn/dc Is the specific refractive index increment of the protein solution. In fact the value of dn/dc is rather uniform for proteins in aqueous solutions, an average value of 0.185×10^{-3} 1/g is taken.

k Is a constant which depends on the intrinsic characteristics of the apparatus.

dx Expressed in arbitrary units corresponds to the displacement of the Young fringes when replacing the protein solution by the solvent medium
So that:

$$A^{1\%}_{1cm} = \frac{\Delta DO}{k \cdot \Delta x} \cdot \frac{dn}{dc} \cdot 10$$

Table 1 gives the different $A^{1\%}_{1cm}$ values. The average values are:

277 nm $A^{1\%}_{1cm} = 3.72 \pm 0.04$.

280 nm $A^{1\%}_{1cm} = 3.28 \pm 0.04$.

These values are significantly lower (20%) than those given by Talalay [2] and still used very recently by other authors [18].

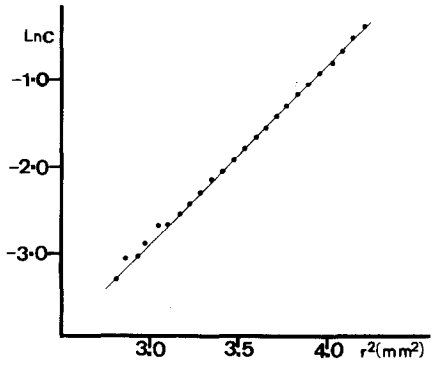

Fig. 1. Equilibrium sedimentation analysis of pure isomerase 0.3 mg/ml in potassium phosphate buffer 0.03 M, pH 7 performed at 15°C and 40 000 rpm for 48 hr. The apparent mol. wt is obtained from the slope of the straight line:

$$\mathrm{Ln}C = \frac{M_{app.}\,(1-\bar{v}\rho)\omega^2}{2RT} \cdot r^2$$

3.2. *Molecular weight determination*

3.2.1. Light scattering

The molecular weight of isomerase was determined from the series of straight lines obtained by plotting I_θ at different protein concentrations (c in mg/ml) versus $\sin^2 \theta/2$. $^cI_\theta$ is the scattered light intensity at a given angle value θ. The different extrapolated values I_θ/c at $\sin^2(\theta/2) = 0$ for each protein concentration were graphed versus protein concentrations giving at zero concentration a mol. wt of 25 300 ± 2000.

3.2.2. By ultracentrifugation equilibrium

Using Yphantis representation a mean mol. wt of 26 000 ± 1 800 was determined from the slopes of the straight lines obtained by plotting log C versus $r^2(mm^2)$. C is the absolute concentration at any point and is equal to the concentration difference between that point and the meniscus; r is the distance to the rotor axis at each point. Fig. 1 shows such a representation for initial protein concentration of 0.3 mg/ml. The good linearity of the points confirms the homogeneity of the preparation.

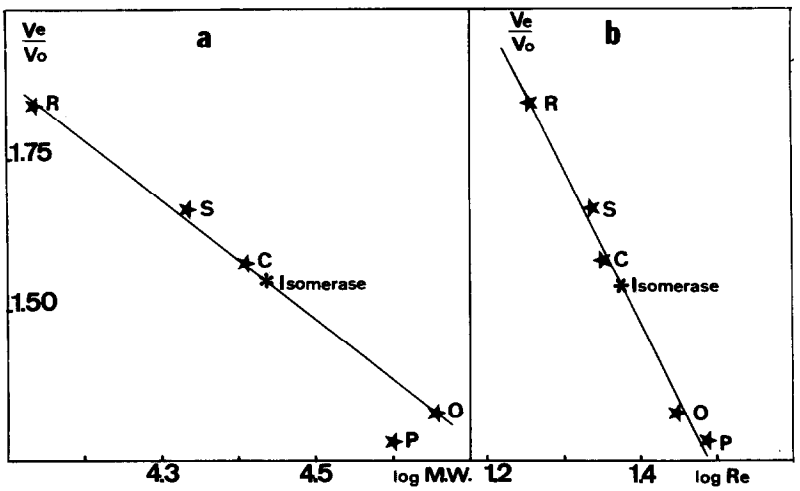

Fig. 2. Sephadex G-100 filtration: (a) correlation of V_e/V_o with mol. wt. The gel filtration data (V_e/V_o) of table 2 are plotted versus log of mol. wt. Experimental conditions are previously described. Abbreviations: R (ribonuclease); S (soybean trypsin inhibitor); CH (chymotrypsinogen); P (peroxidase); O (ovalbumin); (b) correlation of V_e/V_o with Stokes radii. Gel filtration data (V_e/V_o) of table 2 are plotted versus log of mol. wt. Experimental conditions are previously described; for abbreviations see 2(a).

3.2.3. The chromatography experiments on Sephadex G-100

The experiments [19,20] gave a single V_e/V_o value of 1.54 at four different protein concentrations from 0.003 to 0.5 mg/ml (ionic strength, $\mu = 0.11$), one V_e/V_o determination was carried out at $\mu = 0.17$.

Fig. 2a shows the variation of V_e/V_o as a function of log of molecular weight. V_e is the elution volume of the different proteins, V_o is the void volume. There is a satisfactory linear relationship between V_e/V_o and log of molecular weight except for peroxidase the behavior of which is anomalous, this can be easily

Table 2
Molecular parameters of standard proteins and isomerase.

Proteins	Mol. wt	Stokes radius (R_e in Å)	f/f_o	V_e/V_o	References
Ribonuclease	13 700	18.0	1.05	1.84[a]	21
Soybean trypsin inhibitor	21 500	21.9	–	1.67[a]	22
Chymotrypsinogen	25 600	22.5	1.11	1.58[a]	21
Isomerase	27 800[a]	23.6 ± 0,2[a]	1.18[a]	1.54[a]	
Peroxidase	40 000	30.2[b]	1.54	1.28[a]	23
Ovalbumin	45 000	27.6	1.08	1.33[a]	21

[a] This work
[b] The $R_e \simeq 30$ Å was calculated from:

$$f = 6\pi\eta R_e$$

$$R_e = \frac{kT}{D^0_{20}\, 6\pi\eta} \quad [24]$$

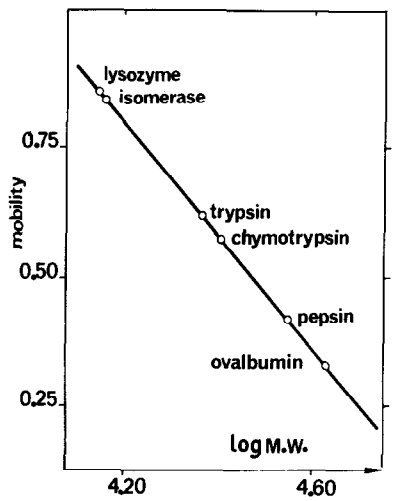

Fig. 3. Subunit molecular weight of isomerase in 1% sodium dodecyl sulfate gel electrophoresis. Electrophoretic mobilities of different protein markers and isomerase are plotted versus log of mol. wt.

explained by its high f/f_0 ratio (1.54) that we have calculated from data given in the literature [12] according to Tanford [24]. The estimated molecular weight of isomerase obtained from this linear graph is 27 000 ± 1 000.

In fig. 2b is shown the variation of V_e/V_o as a function of Stokes radii (R_e in Å). There is a good linear correlation between V_e/V_o and log of R_e. This is in agreement with the data given by Squire [20]. From this straight line an R_e value of isomerase is determined; it is equal to 23.6 ± 0.2 Å.

Table 2 shows molecular parameters of standard proteins and V_e/V_o ratios obtained in this work with these proteins and isomerase. From these three independent techniques a very satisfactory reproducible molecular weight of isomerase is obtained, it varies between 25 300 and 27 600. One may therefore conclude that isomerase is a smaller molecule than the oligomer of 40 800 described by Talalay.

3.3. *Subunit molecular weight*

Fig. 3 shows the variation of the electrophoretic mobilities of the standard markers and of isomerase versus log of molecular weight. The isomerase mobility is very close to that of lysozyme, giving a mol. wt of 14 000 for the subunit polypeptide chain. When

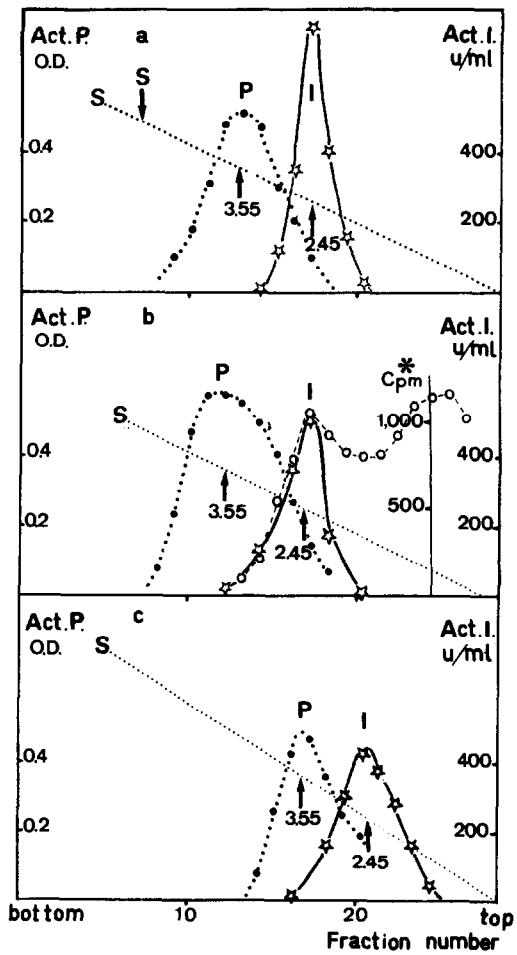

Fig. 4. Sucrose density ultracentrifugation gradient of isomerase. A 5–10% sucrose gradient is prepared in phosphate potassium buffer 0.03 M, pH 7. Centrifugation is carried out in a SW 50 rotor at 55 000 rpm for 20 hr. Isomerase is 10^{-5} M in the 0.1 ml layered on top of the gradients: (a) Free isomerase (BSA and peroxidase are reference proteins); (b) Labelled isomerase with 4-[^{14}C]nortestosterone (Peroxidase is the reference protein); (c) Free isomerase in D_2O (peroxidase is the reference protein); (☆—☆—☆) isomerase activity; (●—●—●) peroxidase activity; (○—○—○) radioactivity.

using ribonuclease as a low molecular weight marker, a value of 12 000 was obtained. The discrepancy of these two values is not surprising knowing the abnormal behavior in SDS gel electrophoresis [25] of small polypeptide chains (mol. wt < 15 000). Comparing the average subunit mol. wt (13 000 ± 10%) with the oligomer mol. wt (≃ 26 300) obtained by the different techniques in this work, it is suggested that iso-

merase is a dimer formed of two polypeptide chains of identical molecular weight.

3.4. *Sedimentation coefficient $s^0_{20,w}$ in sucrose density gradients.*

3.4.1. $s^0_{20,w}$ of isomerase in the absence and in the presence of steroid inhibitors.

Fig. 4a shows the sedimentation profiles of isomerase and peroxidase used as an internal marker. According to Martin and Ames [14] an $S^0_{20,w}$ value of 2.45 S is determined (isomerase concentration 3.6×10^{-5} mg/ml). From a Schlieren pattern by analytical ultracentrifugation with isomerase at 3.3 mg/ml a 2.4 S value is obtained.

Fig. 4b shows a sucrose gradient profile of isomerase with 19-nortestosterone, using peroxidase as an internal marker. As may be seen, the peak of radioactivity corresponding to the steroid–isomerase complex is superimposable with the isomerase activity peak giving also a 2.45 S value. Half of the total radioactivity remains unbound as expected from experimental conditions. An identical S value was determined with estradiol (another competitive inhibitor of isomerase).

The unchanged $s^0_{20,w}$ value indicates that no variation occurs in the polymerisation degree of isomerase when bound to steroid and that the dimer is the molecular active form.

3.4.2. Estimation of the partial specific volume ($\bar{v}$) of isomerase

The $\bar{v}$ of isomerase was determined from $s^0_{20,w}$ measurements either in H_2O or in D_2O according to Meunier et al. [26]. Peroxidase [12] was used as a reference protein ($s^0_{20,w} = 3.55\ s, \bar{v}_0 = 0.699$ ml/g). As recommended by Bon et al. [27] $\bar{v}$ has been calculated from equations:

$$r = \frac{s'_{app.}}{s_{app.}} = \frac{k - \bar{v}\rho'}{k - \bar{v}_0\rho'} \cdot \frac{1 - \bar{v}_0\rho}{1 - \bar{v}\rho}$$

$s_{app.}$ and $s'_{app.}$ are respectively the apparent sedimentation coefficients of isomerase in H_2O and D_2O, both determined with respect to the $s^0_{20,w}$ value of peroxidase; $\bar{v}_0$ and $\bar{v}$ are respectively the partial specific volumes of peroxidase and isomerase; k corresponds to the mass variation of the protein due to proton–deuteron exchange; it is considered uniform for all proteins ($k = 1.0155$). ρ and ρ' are the corresponding densities of H_2O ($\rho = 1$) and D_2O ($\rho' = 1.105$) at 4°C. These values have been considered as those of low ionic strength media ($\mu = 0.066$).

Fig. 4c shows the sedimentation profiles of isomerase in H_2O and D_2O.

In H_2O: $s^0_{20,H_2O} = 2.45$ S

In D_2O: $s^0_{20,D_2O} = 2.35$ S

From these experimental data $\bar{v} = 0.738$ ml/g. This $\bar{v}$ value is very close to 0.745 ± 0.150 ml/g calculated previously [28] according to Cohn and Edsall [29].

3.4.3. Calculation of molecular parameters of isomerase

The molecular parameters f/f_0 and $d_{20,w}$ are calculated according to Siegel and Monty equations [30].

$$f/f_0 = \frac{R_e}{(3\bar{v}\ \text{mol. wt}/4\pi\eta N)^{1/3}}$$

$$D = \frac{kT}{6\pi\eta D R_e}$$

$f/f_0 = 1.18$, $D_{20°C} = 9.1 \times 10^{-7}$ cm^2/sec.

Almost identical $D_{20,w}$ (8.55×10^{-7} cm^2/sec) value was obtained when using the equation:

$$\text{mol. wt} = \frac{s^0_{20,w}}{D} \cdot \frac{RT}{(1 - \bar{v}\rho)}$$

As may be seen from the f/f_0 ratio, isomerase behaves as a globular protein, this is supported by the good correlation between the elution volumes and the log of mol. wt of an extensive series of globular proteins used as markers (fig. 2a).

3.5. *Isoelectric point of isomerase*

The pI obtained is equal to 4.75, such an acidic pI cannot be easily explained by the amino acid composition of isomerase [4].

In summary, isomerase, under the present experimental conditions, when enzymatically active and able to bind competitive inhibitors, is a 26 300 mol.

wt dimer formed of two polypeptide chains of identical mol. wt ($\simeq$ 13 000 ± 10%). Furthermore, spectrofluorimetric and circular dichroic studies (to be published) of the number of binding sites, have shown that one mole of steroid is bound per dimer (26 300 mol. wt). The mechanism of this unusual binding is under investigation.

Acknowledgements

This work has been partially supported by the Ford Foundation and the C.N.R.S. *Pseudomonas testosteroni* extracts and various steroids were given by Roussel-UCLAF.

The authors thank Professor Jacques Tonnelat and Mrs Andrée Barnéoud for $s^0_{20,w}$ determinations of BSA by analytical ultracentrifugation, and Dr. Sylvanie Guinand for the helpful assistance for the $A^{1\%}_{1cm}$ estimation by refractometry. They express sincere thanks to Dr. Dominique Pantaloni and his colleagues for skillful technical assistance and helpful advice in Yphantis ultracentrifugation and light scattering studies.

The authors thank kindly also Mrs. Anne Atger for secretarial assistance.

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