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Crystallization and preliminary crystal structure of the complex of 17β-hydroxysteroid dehydrogenase with a dual-site inhibitor

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Abstract

Human estrogenic 17β-hydroxysteroid dehydrogenase (17β-HSD1) catalyzes the synthesis of 17β-estradiol (E_2) from estrone, in the ovary and peripheral tissues. While the structures of 17β-HSD1 alone and in complex with E_2 have been determined (D. Ghosh, V. Pletnev, D.-W. Zhu, Z. Wawrzak, W.-L. Duax, W. Pangborn, F. Labrie, S.-X. Lin, Structure of human 17β-hydroxysteroid dehydrogenase at 2.20 Å resolution, Structure 3 (1995) 503–513), no structures of inhibitor/enzyme complex, either modeled or from crystallography, have been reported before the submission of the present paper. The best available inhibitors are among the 'dual-site inhibitors', blocking estrogenic 17β-HSD and the estrogen receptor. These compounds belong to a family of estradiol analogues having an halogen atom at the 16α position and an extended alkyl-amide chain at the 7α position (C. Labrie, G. Martel, J.M. Dufour, G. Levesque, Y. Merand, F. Labrie, Novel compounds inhibit estrogen formation and action, Cancer Res. 52 (1992) 610–615). We now report the crystallization of this enzyme/inhibitor complex. The complex of the best available dual-site inhibitor, EM-139, with 17β-HSD1 has been crystallized using both cocrystallization and soaking methods. Crystals are isomorphous to the native crystals grown in the presence of 0.06% β-octyl-glucoside and polyethyleneglycol 4000, with a monoclinic space group C2. Data at 1.8 Å have been collected from a synchrotron source. Even though the size of the inhibitor is greater than that of the substrate, our preliminary X-ray-diffraction study shows that EM-139 fits into the active site in a position similar to that of estrogen. The availability of such structural data will help design more potent inhibitors of estrogenic 17β-HSD.

Keywords: Protein; Crystallization; Inhibitor; Structure; Estradiol

1. Introduction

Since human estrogenic 17β-hydroxysteroid dehydrogenase (17β-HSD1, EC1.1.1.62) expressed in breast cancer tissue is responsible for the formation of the most active estrogen, estradiol [4,5], it becomes one of the targets for breast cancer therapy (see end of the text). In fact, since elucidation of the structure of its cDNA and its gene [5,6], many studies have been devoted to the control of 17β-HSD1 activity (see [7] for review) and the development of inhibitors. Some affinity label inhibitors for human placental 17β-HSD1 have been reported [8–13]. Two characteristics of these compounds, namely their low selectivity and their unsuitable estrogenic activity, virtually eliminated their therapeutic use [14–16].

Recently, a series of dual-site inhibitors (EM-139, EM-221, EM-140, and EM-123) have been synthesized in our laboratory (Fig. 1) [3,17]. These compounds possess an estrogen nucleus and can block the formation of active estradiol by type 1 17β-HSD, as well as the action of estradiol via its receptor, while possessing no intrinsic estrogenic activity.

EM-139 is a 7α-alkyl, 16α-halo estradiol derivative [3,17] with pure antiestrogenic activity. In order to optimize the enzyme-inhibitor interactions, it is im-

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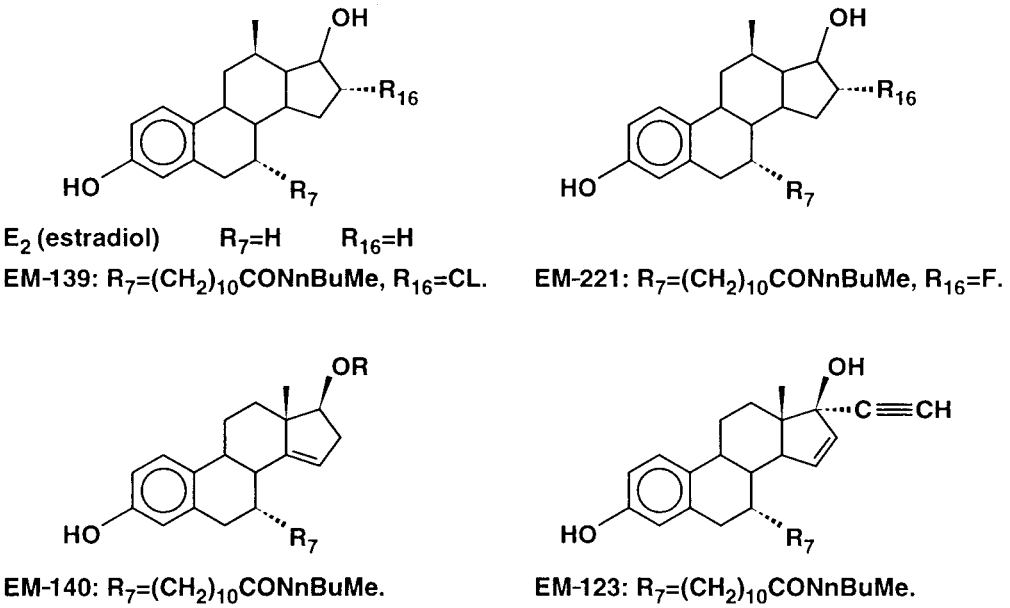

Fig. 1. Structure of representative novel compounds acting as pure antiestrogens and inhibitors of 17β-HSD1 activity. 16α-halogenated compounds: EM-139 and EM-221. D-ring unsaturated compounds: EM-140, and EM-123 [3].

portant to gain a better understanding of the crystal structure of this inhibitor with 17β-HSD1. This compound was tested for its antiestrogenic activity as well as its potential 17β-HSD1-inhibitory activity in ovariectomized mice treated with estrone, the immediate precursor of E_2 [3]. More recently, kinetic studies have shown that EM-139 is a reversible and competitive inhibitor for 17β-HSD1 (Lin et al., unpublished).

We successfully crystallized 17β-HSD1 [18], and its structure was thus determined at 2.2 Å-resolution [1]. Recently, we have crystallized the complex of 17β-HSD1 with estradiol (17β-HSD1-E_2), and its structure has been determined at 2.3 Å-resolution [2], and the structure of 17β-HSD1 with estradiol and $NADP^+$ has also been reported [19]. No structures, either modeled or from crystallography, have been reported for the dual-site inhibitors bound to this enzyme. Here we report the complex of 17β-HSD1 with the best dual-site inhibitor available, EM-139, crystallized via both cocrystallization and soaking.

2. Materials and methods

2.1. Chemicals

NAD^+, glycerol, β-OG (β-octyl glucoside), $MgCl_2$, $NaHCO_3$-Na_2CO_3, PEG (polyethylene glycol) 4000, Tris-base [Tris = tris-(hydroxymethyl)aminomethane], Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonicacid], EDTA (ethylenediaminetetraacetic acid), DTT (dithiothreitol) and PMSF (phenylmethanesulfonyl fluoride) were purchased from Sigma Aldrich Canada (Oakville, Ont.). The dual-site inhibitor, EM-139, was synthesised in our laboratory (Laboratory of Molecular Endocrinology, Laval University Medical Research Centre) [3,17]. Q-Sepharose Fast Flow and Blue-Sepharose CL-6B and phenyl superose columns were packed in our laboratory using media obtained from Pharmacia Biotech. (Montreal). All reagents were of the best grade available. Celltricon-30 and Centriprep-30 concentrators were purchased from Amicon (Beverly, MA, USA).

2.2. Assay of 17β-HSD1

Measurement of 17β-HSD1 activity was performed as described previously [20]. When the enzyme was assayed by spectrophotometry, the reduction of NAD^+ was measured by observing the absorbance increase at 340 nm and 23 ± 1°C. The reaction mixture contained 25 μM estradiol, 0.5 mM NAD^+ in 50 mM $NaHCO_3$ buffer, pH 9.2. At this pH, maximum activity of estradiol oxidation is obtained. A blank value lacking estradiol was measured under the same conditions and was subtracted.

$$\text{Estradiol} + NAD^+ \overset{17\beta-HSD1}{\rightleftharpoons} \text{Estrone} + NADH + H^+$$

One unit of enzyme is defined as the amount of enzyme that catalyzes the formation of one μmol of product in one min under the above-indicated conditions.

2.3. Purification of 17β-HSD

17β-HSD1 is located in the soluble subcellular fraction of human placenta. A Pharmacia FPLC (Fast Protein Liquid Chromatography) system was used for rapid purification of the enzyme, as described previously [18,20]. The purified human 17β-HSD1 thus obtained showed a high specific activity at room temperature: formation of approximately 8 μmol of estrone from estradiol per min per mg enzyme protein, at pH 9.2.

2.4. Determination of the protein concentration

The concentration of 17β-HSD1 was determined by the Bradford method [21], from the absorption at 595 nm, using BSA (bovine serum albumin) as a standard. The optical density was measured with a Beckman DU-70 spectrophotometer.

2.5. Preparation of apoenzyme and 17β-HSD1-EM-139 complex

The enzyme sample was collected from the phenyl-Sepharose chromatography column and prepared in a buffer containing 20% glycerol, 1 mM EDTA, 0.4 mM DTT, 0.5 mM PMSF, and 40 mM Tris–HCl, pH 7.5, hereafter referred to as buffer A. Before crystallization, β-OG (0.06%) was added to the apoenzyme sample (<12 μM) by centricon buffer change. The sample was then centrifuged at 4500 *g* in a Sorvall RC-5 centrifuge and the final concentration was 15 mg/ml. Formation of the 17β-HSD1-EM-139 complex was shown by a spectroscopic method. A parallel binding assay was used to measure EM-139 using a Beckman DU-70 spectrophotometer at 280 nm. After three changes of buffer, (buffer A, containing 0.06% β-OG and 25 μM EM-139), a stoichiometric 17β-HSD1-EM-139 complex was obtained as verified by measuring the absorbance at 280 nm.

2.6. Crystallization of 17β-HSD-EM-139

The cocrystallization experiment was carried out using the vapor diffusion method at room temperature with polyethyleneglycol (4000, 26% w/v) as the precipitant. The reservoir contained 0.15 M $MgCl_2$ and was buffered to pH 7.5. The hanging drop was formed by 3 μl of the concentrated complex solution (15 mg/ml) and 3 μl of the solution from the reservoir. The soaking method was based on crystallizing the apoenzyme using the above conditions. When the enzyme crystals stopped growing (about 4–6 weeks), 1 mM EM-139, which is soluble only in the presence of PEG, was added to the drop.

2.7. Crystal characterization, data collection and analysis

The X-ray diffraction analysis of crystals of 17β-HSD1-EM-139 was performed using an R-axis-IIc image plate detector on a Rigaku RU200 rotating anode with a normal focus 0.5×10 mm filament. Data were also collected using a MAR 30 cm image plate at beam line X12C at the National Synchrotron Light Source, Bookhaven National Laboratory, Upton, NY. Data obtained on the R-axis Ilc were collected at ambient temperature (~23°C) with the crystals mounted in sealed glass capillaries. The R-axis Ilc detector was 100 mm from the crystal and the beam was collimated to 0.5 mm. Data collection at the synchrotron was performed at a temperature of 123 K with the detector 197 mm from the crystal. The wavelength used was 1.15 Å. In both cases, data were collected using the oscillation method (1.5° oscillations) about the ψ axis. At least 58° worth of data were collected on the *r*-axis Ilc from each crystal before excessive radiation-induced decay was observed. A full 175.5° of data were collected with the synchrotron. All data sets were processed with the HKL software package.

3. Results and discussion

3.1. Preparation of 17β-HSD1-EM-139 complex

Using fast protein liquid chromatography (FPLC), 17β-HSD1 possessing high specific activity and homogeneity was obtained. The high quality of the enzyme protein thus obtained, is in fact important in the successful crystal growth of 17β-HSD1.

The 17β-HSD1 preparation possesses a solubility of 2–3 mg/ml in the absence of detergent. When the mild detergent β-OG was added to a final concentration of 0.06%, 17β-HSD1 solubility was increased to >40 mg/ml. The nonionic detergent plays a role in reducing nonspecific hydrophobic interactions [22].

As mentioned above, EM-139 belongs to a family of 7α-alkyl, 16α-halo derivatives of estradiol that act as dual-site inhibitors of both estrogen formation by 17β-HSD1 and action on the estrogen receptor [3]. Based upon knowledge gained during preparation of the 17β-HSD1-E2 complex [23], we have used a special procedure to saturate the enzyme at a high concentration with the inhibitor. Before crystallization, formation of the 17β-HSD1-EM-139 complex was verified by a spectrophotometric method [24]. It is well known that steroids have very low aqueous solubility. In fact, under our experimental conditions, at room temperature, about 30 μM of EM-139 could be solubilized in an aqueous solution. When gradually saturating the enzyme with EM-139 starting at a low 17β-HSD1 con-

Fig. 2. Cocrystallization: crystals of 17β-HSD1-EM-139. The crystals were obtained in the presence of 20% glycerol, 0.16 M Mg Cl_2, 0.1 M Hepes, pH 7.5 and 26% (w/v) PEG (4000) as the precipitant. Final protein concentration was 15 mg/ml, the total EM-139 concentration being more than 440 μM in the complex. Crystal grew to 0.375 × 0.27 × 0.15 mm in three weeks.

centration, we observed that the total EM-139 concentration could be raised above its solubility, due to its binding to the enzyme.

The 17β-HSD1 concentration used for crystallization was usually higher than 220 μM. If each subunit of 17β-HSD1 is bound with one molecule of EM-139, the total EM-139 concentration should be more than 440 μM in the complex. For example, in one control, before crystallization via centricon buffer change, EM-139 was added to the enzyme sample ($V_1 + V_2 = 2.5$ ml) and the concentration ($[\text{EM-139}]_0$) in this initial mixture was 25 μM. Measuring the absorbance at 280 nm, we could find that the absorption of the sample buffer was 0.3995 ($[\text{EM-139}]_0$). After centricon concentration for 60 min, the volume of 17β-HSD1-(EM-139) sample was decreased by more than 78-fold to 32 μl (V_s). The absorption of filtrate buffer ($V_F = 2.468$ ml) was decreased to 0.2588, so that this buffer contained 16 μM EM-139 ($[\text{EM-139}]_F = 25\ \mu\text{M}/0.3995 \times 0.2588 = 16\ \mu\text{M}$). The high concentration of the 17β-HSD1-(EM-139) sample can be expressed by the following equation: $(V_1 + V_2) \times [\text{EM-139}]_0 = V_S \times [\text{EM-139}]_S + V_F \times [\text{EM-139}]_F$, where $V_1 + V_2$ is the initial volume for 17β-HSD with EM-139 before centrifugation, and V_S and V_F, are the sample and filtrate volumes, respectively, after centricon concentration. The results obtained indicate that 17β-HSD1 is complexed with EM-139 (>700 μM). The hydrophobic EM-139 was bound to the hydrophobic binding pocket of 17β-HSD1 and it saturated the latter in a special repeated 'dialysis', thus gradually reaching stoichiometric binding to the enzyme.

3.2. Cocrystallization and soaking

Based upon the former results of crystallization obtained with other 17β-HSD1 forms [18,23], further refinement was carried out with the PEG/$MgCl_2$/β-OG system. A series of crystals of 17β-HSD1-EM139 were obtained with different PEG (4000) and protein concentrations, but the best ones were obtained in the presence of 0.16 M $MgCl_2$, 26% (w/v) PEG (4000) and 0.1 M Hepes pH 7.5, with 15 mg/ml protein (with 0.06% β-OG) as the final concentration after equilibrium. Single crystals appeared within 48 h at room

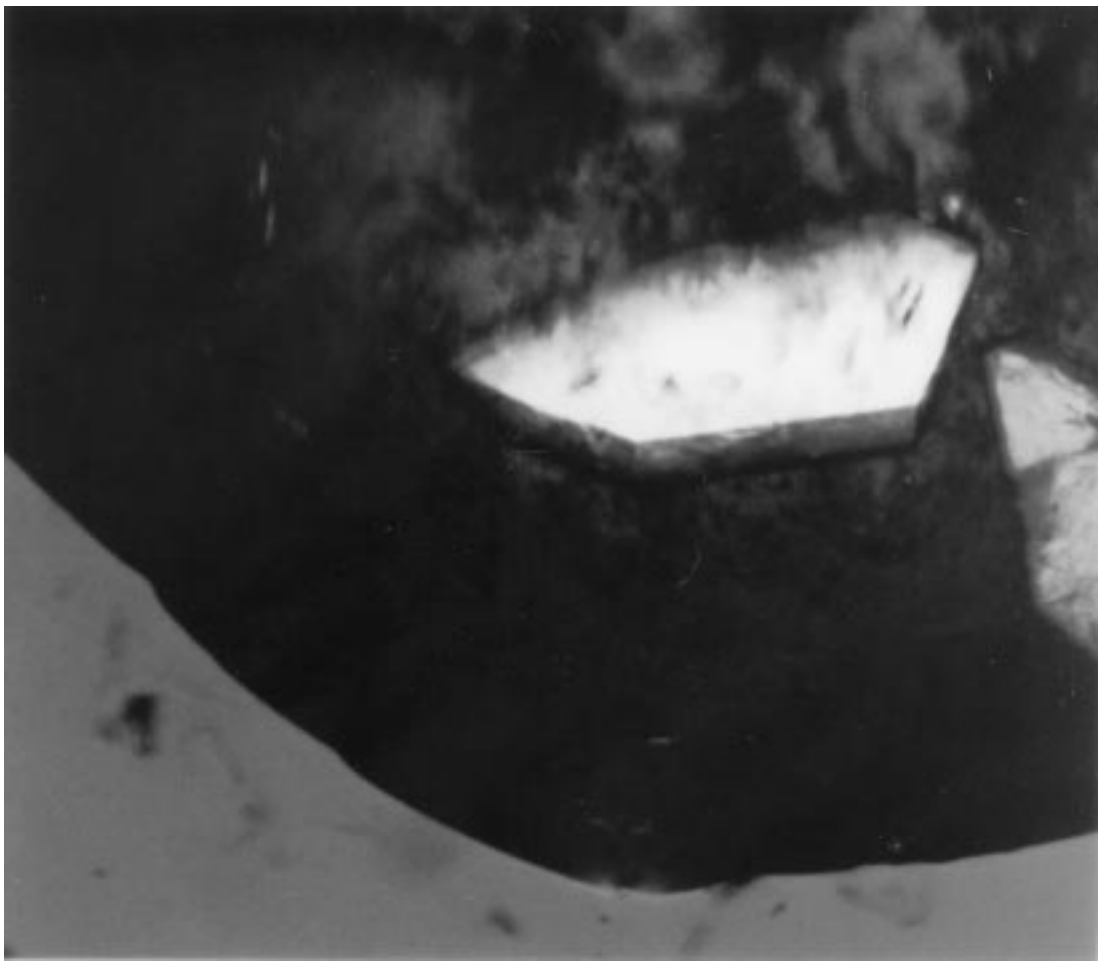

Fig. 3. Soak method: crystals of 17β-HSD1-EM-139. The crystals of apoenzyme were obtained in the presence of 26% (w/v) PEG (4000), 0.15 M $MgCl_2$, 20% glycerol and 0.1 M Hepes, pH 7.4. Final protein concentration was 15 mg/ml. After six weeks, the EM-139 solution was added to drops (the final concentration was 1 mM). The crystals were soaked for ten days. The crystal size is $0.675 \times 0.255 \times 0.15$ mm.

temperature and these grew to a typical size of $0.375 \times 0.27 \times 0.15$ mm^3 in about three weeks (Fig. 2).

Using the PEG/$MgCl_2$/β-OG system, some crystals of apoenzyme were obtained in the presence of 0.15 M $MgCl_2$, 26% (w/v) PEG (4000), 0.06% β-OG, and 0.1 M Hepes pH 7.4, with 15 mg/ml protein as the final concentration after equilibrium. In six weeks, single crystals grew to a typical size of $0.675 \times 0.255 \times 0.15$ mm^3 (Fig. 3). The EM-139 solution was added to the drop (the final concentration was 1 mM) and the crystals were soaked for 10 days. Though EM-139 has very limited solubility in water, 1 mM EM-139 could be dissolved in the presence of 26% PEG (4000) and 20% glycerol.

The preliminary analysis of crystals obtained with the above-described two methods of crystallization shows that the electronic density corresponding to inhibitor (EM-139) bound to the enzyme could be re-

Table 1
Data collection

Data set with	R-axis: crystal with cocrystallization	R-axis soaked crystal	Synchrotron: soaked crystal
Unit cell			
a (Å)	123.86	123.17	122.30
b (Å)	45.09	44.87	43.64
c (Å)	61.61	60.95	60.43
β (°)	99.16	99.38	99.58
Resolution limit (Å)	2.1	2.1	1.8
Data completeness (%)	58	47	96
Mosaicity	0.76	0.71	0.93

liably identified. The above-described methods should be useful for obtaining complexes of other steroid-converting enzymes with their inhibitors or substrates.

3.3. Preliminary X-ray results

The crystals of the 17β-HSD1-EM-139 complex grow in the space group C2 and are isomorphous to those of the enzyme and of the 17β-HSD1-E_2 complex. Two data sets were collected at room temperature on the rotating anode X-ray source: one using a crystal of the cocrystallized complex and one using a crystal of the enzyme soaked in EM-139. Though neither set was complete, they were integrated, scaled and merged using the HKL program package [25], to determine the mosaicity present in each crystal type. The results are summarized in Table 1. For the two examples tested, there was no significant difference in mosaicity between the cocrystallized and the soaked crystals.

A third complete data set was collected on the synchrotron source (Table 1). The higher mosaicity in this case may be a consequence of the flash freezing of the crystal in the liquid N_2 cold stream. This data set was again integrated, scaled and merged and Fourier difference maps were calculated using phases calculated from the enzyme structure. Significant electron density of the inhibitor was visible in the active site, superimposed on the position of E_2 in the structure of the 17β-HSD1-E_2 complex. From prior attempts at computer graphics modeling of the binding of EM-139 to 17β-HSD1 in our laboratory (Campbell et al., unpublished), it is apparent that although the binding site is highly complementary to the steroid core, the long alkyl side chain is apparently accommodated by the flexible loop (residues 190–200) which is not well defined in any of the published 17β-HSD1 structures.

Once this structure is fully refined, analysis of enzyme-inhibitor interactions should permit prediction of the structure of more potent inhibitors which would utilize all possible binding energies. The resulting inhibitor may thus contribute to the improvement of breast cancer therapy. We would like also to mention that aromatase inhibitors obliterate the synthesis of the estrogens efficiently [26,27], while the 17β-HSD1 inhibitors reduce the formation of both estradiol and Δ5-diol, the latter was also found to possess some estrogenic activity [28]. So the inhibition of both aromatase and 17β-HSD1 may further improve the breast cancer therapy.

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