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Two non-reactive ternary complexes of estrogenic 17β-hydroxysteroid dehydrogenase: crystallization and preliminary structural analysis

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Abstract

Human estrogenic 17β-hydroxysteroid dehydrogenase (17β-HSD1, EC1.1.1.62) is an important enzyme that catalyses the last step of active estrogen formation. 17β-HSD1 plays a key role in the proliferation of breast cancer cells. The three-dimensional structures of this enzyme and of the enzyme-estradiol complex have been solved (Zhu et al., 1993, J. Mol. Biol. 234:242; Ghosh et al., 1995, Structure 3:503; Azzi et al., 1996, Nature Struct. Biol. 3:665). The determination of the non-reactive ternary complex structure, which could mimic the transition state, constitutes a further critical step toward the rational design of inhibitors for this enzyme (Ghosh et al. 1995, Structure 3:503; Penning, 1996, Endocrine-Related Cancer, 3:41).

To further study the transition state, two non-reactive ternary complexes, 17β-HSD1–EM519-$NADP^+$ and 17β-HSD1–EM553-$NADP^+$ were crystallized using combined methods of soaking and co-crystallization. Although they belong to the same C2 space group, they have different unit cells, with $a=155.59$ Å, $b=42.82$ Å, $c=121.15$ Å, $\beta=128.5°$ for 17β-HSD1–EM519-$NADP^+$, and $a=124.01$ Å, $b=45.16$ Å, $c=61.40$ Å, $\beta=99.2°$ for 17β-HSD1–EM553-$NADP^+$, respectively. Our preliminary results revealed that the inhibitors interact differently with the enzyme than do the natural substrates.

1. Introduction

17β-hydroxysteroid dehydrogenases (17β-HSDs) constitute a family of isoenzymes that can be found in all classical steroidogenic tissues and most peripheral tissues [1]. They catalyze the oxidation and reduction of steroid hormones. Among this family, 17β-HSD type 1 has been well studied. In breast tumours, 17β-estradiol (E_2) was found in significantly higher concentration than in normal breast tissues [2,3]. Human 17β-HSD1, which is responsible for the formation of the potent estrogen estradiol (E_2) from estrone (E_1) [4], has high activity in malignant breast tissues. Thus, this enzyme is an important target for inhibitor design and breast cancer therapy.

After the determination of the first 3-D crystallographic structure for 17β-HSD1 [5], the structure–function relationship of the enzyme was much better understood, which in turn stimulated further studies of the enzyme structure. The crystallographic studies on this enzyme have been focusing on two main aspects. One aspect is the determination of structural details using site-directed mutagenesis. For example, the importance of His^{221} to the catalytic activity of 17β-HSD1 was demonstrated by the kinetic study of the mutated enzyme [6]. Then this result was proved by the crystal structures of H221L 17β-HSD1 mutant/$NADP^+$ and estradiol complexes, the H221L mutant/NAD^+, and the H221Q mutant/estradiol complexes [7].

The other and more direct aspect consists in study-

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ing the non-reactive ternary complexes, which is a more reliable method for mimicking the transition state and may directly contribute to rational inhibitor design. So far, the available 17β-HSD1-substrate/inhibitor complex structures are binary complexes or reactive ternary complexes [8]. We believe that a structure of a non-reactive ternary complex, which can mimic the transient state and can reveal the detailed interactions between the two partners, would be very helpful to the rational design of inhibitors [9].

In our laboratory, several kinds of inhibitors were synthesized recently, including a series of dual-site action inhibitors [10,11] and other inhibitors [12–14]. These inhibitors, as analogues of steroids, allow the formation and crystallization of non-reactive ternary complexes. The structure of these complexes may in turn give us important information on the ability of the above inhibitors. Here we report the crystallization and preliminary structural analysis of two non-reactive ternary complexes, 17β-HSD1–EM553-$NADP^+$ and 17β-HSD1–EM519-$NADP^+$.

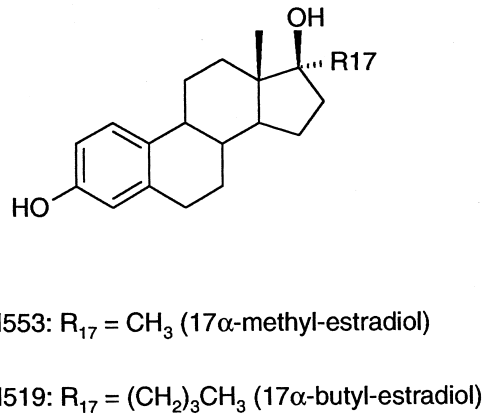

Fig. 1. Structure of EM553 and EM519.

2. Materials and methods

2.1. Apparatus and chemicals

Fast Protein Liquid Chromatography (FPLC), consisting of two P3500 pumps, an UV-M monitor and a LCC-500 controller, was bought from Pharmacia Biotech (Montreal, Canada). Rigaku R-AXIS IIc and Rigaku RU-2HR X-ray generators were used for data collection. Synchrotron data collection was carried out at Cornell High Energy Synchrotron Source (CHESS) with a CCD detector at beamline A1.

Dithiothreitol (DTT), glycerol, β-OG (β-octyl glucoside), $NADP^+$, LiCl, NaCl, $NaHCO_3$–Na_2CO_3, PEG (polyethylene glycol) 3500, Tris–base [Tris–(hydroxymethyl) aminomethane], Hepes [*N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid], EDTA (ethylenediaminetetraacetic acid), PMSF (phenylmethylesulfonyl fluoride) and progesterone were purchased from Sigma (St. Louis, MO, USA). Blue-Sepharose CL-6B and Phenyl-Superose HR 10/10 columns were packed in our laboratory using media from Pharmacia Biotech (Montreal, Canada), and Mono-Q HR 5/5 column was bought from the same company. Centricon-30 concentrators were bought from Amicon (Beverly, MA, USA).

2.2. Methods

The two inhibitors, EM553 (17α-methyl-17β-estradiol) and EM519 (17α-butyl-17β-estradiol), were synthesized by adding an excess of methyllithium or n-butyllithium to estrone, and purified with a silica gel chromatography by the Chemistry group in our Laboratory.

The recombinant enzyme protein expressed in Sf-9 insect cells was purified with a Pharmacia FPLC system using a rapid purification procedure consisting of two chromatographic steps: Blue-Sepharose affinity and Phenyl-Superose hydrophobic interaction columns [15,16]. However, we added an ion-exchange column, Mono-Q HR 5/5, as the last chromatographic step in order to get a more homogeneous protein. During the whole process of purification, as well as the crystallization procedure, 20% glycerol, 0.4 mM DTT, 0.5 mM PMSF, 1 mM EDTA were used to stabilize the enzyme activity of 17β-HSD1.

Crystallization was performed using vapor diffusion technique in hanging drops. The initial condition for growing apo-enzyme crystals was based on our previous work [17]. The ligand stock solutions were introduced to the drops by several small additions.

The crystals of the 17β-HSD1–EM553-$NADP^+$ complex were mounted in glass capillaries. Data collection was performed at room temperature on an R-AXIS IIc image plate detector. A data set was collected through 90° (1.5° oscillation). The data on the 17β-HSD1–EM519-$NADP^+$ complex was collected from beamline A1 at Cornell High Energy Synchrotron Source with a CCD detector at 100 K. Two full data sets of 180° were collected. All data sets were processed with the HKL software package [18].

3. Results

3.1. Pre-crystallization

Homogeneous and highly active 17β-HSD1 was prepared using the procedure described in the Methods with an additional Mono-Q chromatography. A mild non-ionic detergent, β-octyl glucoside (0.06%) was

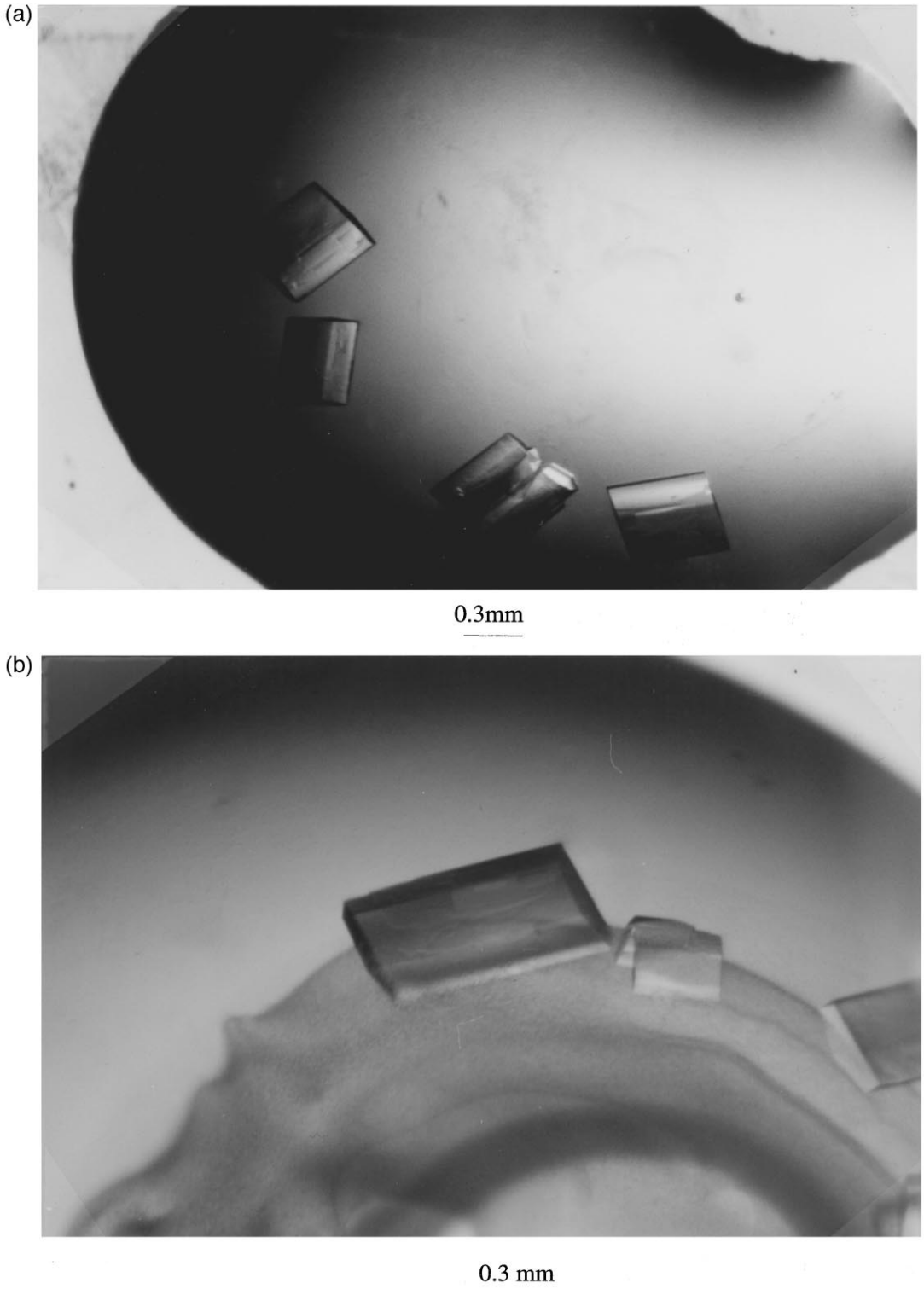

Fig. 2. The crystals of 17β-HSD1–EM553-NADP$^+$ and 17β-HSD1–EM519-NADP$^+$ complexes. (a) 17β-HSD1–EM553-NADP$^+$ crystals were obtained in the presence of 20% glycerol, 0.16 M $MgCl_2$, 0.1 M Hepes, pH 7.5 and 25% (w/v) PEG 4 K. Final enzyme concentration was about 15 mg/ml, EM553 concentration is about 1 mM. The best crystal was about $0.55 \times 0.50 \times 0.18$ mm in size. (b) 17β-HSD1–EM519-NADP$^+$ crystals were obtained in the presence of 15% glycerol, 0.16 M $MgCl_2$, 0.1 M Hepes, pH 7.5 and 27% (w/v) PEG 4 K. Final enzyme concentration was about 15 mg/ml, EM519 concentration is about 1 mM. The best crystal was about $0.70 \times 0.50 \times 0.20$ mm in size.

gradually added to the protein solution by centricon buffer change, thus increasing the enzyme solubility. A final concentration of 15 mg/ml was obtained. In fact, the enzyme preparation can reach 40 mg/ml or more

Table 1
Data collection of 17β-HSD1–EM553-NADP$^+$ complex

X-ray source	Rigaku RU-200
Total frames collected	60
Oscillation angle (°)	1.5
Resolution limit (Å)	50–2.2
Total number of reflections used	48296
Unique reflection number	17221
Redundancy	2
Data completeness (%)	95.5
Mosaicity (°)	0.42
Unit cell	$a=124.01$ Å, $b=45.16$ Å, $c=61.40$ Å, $\beta=99.2°$
Linear R factor	0.046
Square R factor	0.033

in the presence of 0.06% β-OG. Before crystallization, the final preparation was checked by SDS–PAGE analysis and it revealed a single band at 34.5 kDa.

Since the inhibitors, EM519 and EM553 (Fig. 1), are derived from steroids, both of them have very low solubility. In order to guarantee the formation of stoichiometric ternary complexes, the inhibitors were dissolved in ethanol to a high concentration (10 mM) as a stock. In the presence of PEG, a 1 mM aqueous solution of the inhibitors can be obtained. NADP$^+$ was prepared to a concentration of 30 mM in the precipitant solution.

3.2. Crystallization

The optimized crystallization condition for the apo-enzyme was derived from the previous results of our group [17]. However, in the case of the 17β-HSD1–EM519-NADP$^+$complex, 10–15% of glycerol was used in the precipitant solution, instead of 20% as in other experiments. Compared to our previous results, much less precipitant was observed in the drop after mixing the precipitant solution (3 μl) and protein sample (3 μl). In 1 week, two or three small crystals (about $0.2 \times 0.15 \times 0.08$ mm) appeared in the drop. Then, 0.2 μl of NADP$^+$ stock was repeatedly added to the edge of the drop in 4–5 additions. It slowly reached the crystals by liquid–liquid diffusion. At equilibrium, the final concentration of NADP$^+$ in the drop could reach 5 mM. Inhibitors were also added to the drop using the same method. The final concentration of inhibitors was about 1 mM in the presence of PEG. In the presence of ligands in the drops, the crystals continued to grow. They reached a typical size of $0.6 \times 0.5 \times 0.2$ mm in 5–6 weeks (Fig. 2), about 25 times bigger than the volume of starting crystals before the ligand addition.

3.3. Preliminary X-ray results

The crystals of the 17β-HSD1–EM553-NADP$^+$ complex belong to C2 space group. The unit cell $a=124.01$ Å, $b=45.16$ Å, $c=61.40$ Å, $\beta=99.2°$ is similar to that of the apo-enzyme $a=123.03$ Å, $b=45.03$ Å, $c=61.29$ Å, $\beta=99.1°$. A summary of the data collection appears in Table 1.

Two data sets from a 17β-HSD1–EM519-NADP$^+$ crystal were integrated, scaled and merged using the HKL program package [18]. Although it also belongs to C2 space group, its unit cell $a=158.59$ Å, $b=42.82$ Å, $c=121.15$ Å, $\beta=128.5°$ is different from that of other 17β-HSD1 crystals [17]. In fact, the volume of this unit cell is about twice that of the 17β-HSD1–EM553-NADP$^+$ complex. This indicates that there are

Table 2
Data collection of 17β-HSD1–EM519-NADP$^+$ complex

X-ray source	Synchrotron
Total frames collected	361
Oscillation angle (°)	1.0
Resolution limit (Å)	40–1.8
Total number of reflections used	846371
Unique reflection number	58418
Redundancy	7–8
Data completeness (%)	93.2
Mosaicity (°)	0.90
Unit cell	$a=155.89$ Å, $b=42.82$ Å, $c=121.15$ Å, $\beta=128.5°$
Linear R factor	0.080
Square R factor	0.111

two monomers in one asymmetric unit of 17β-HSD1–EM519-NADP$^+$ complex crystals, while there is only one monomer in the other form. The results of the data collection are summarized in Table 2.

4. Discussion

For our study of non-reactive ternary complexes, we selected two 17α-alkyl derivatives of enzyme substrate estradiol (EM553 and EM519), because their tertiary alcohol (OH) cannot be oxidized in the enzymatic process. However, as previously reported [12], the 17β-HSD1 affinity for estrogen decreases markedly by adding an alkyl group at position C17α. EM553 and EM519, at a concentration of 10 μM, were thus found to inhibit (5 and 78%, respectively) the transformation of labeled estrone into labeled estradiol by 17β-HSD1.

For growing non-reactive ternary complex crystals, we used a new method that was developed for the crystallization of 17β-HSD1 complexes. As it is well known, the steroids including both the inhibitors (EM519 and EM553) have very low solubility. Most steroids can attain a concentration between 0.7 to 1 mM in PEG solution (e.g. 20% PEG4 K). In order to be sure that these ligands were bound to the enzyme, we prepared saturated inhibitor solutions in ethanol. Both EM519 and EM553 can reach a 15 mM concentration in ethanol. In previous experiments, these complexes were formed by using only the soaking method. The ligands were added to the drop after the crystals were fully grown. In this case, however, the addition of ligands often caused the big crystals to crack, for example, the crystals of enzyme–testosterone complex. This may be due to the conformation changes in the enzyme when binding with ligands. It may also be caused by the introduction of a relatively large amount of ethanol from the ligand stock solution. On the other hand, in the former mentioned co-crystallization process, stoichiometric enzyme–ligand complex at high concentration was obtained with a gradual concentration [19]. However, the preparation of such a complex was long and required a considerable amount of enzyme. Taking this into account, in this experiment we chose to use a combined method of soaking and co-crystallization (see Method), which provides an ideal condition for growing crystals of 17β-HSD1 complexed with various ligands.

Recently, we obtained another new crystal form of 17β-HSD1 complexes with a space group of $P2_12_12_1$. From the preliminary crystal structure, we concluded that there were two monomers in one asymmetric unit. This phenomenon is quite similar to the important modification of unit cell parameters on the 17β-HSD1–EM519-NADP$^+$ complex. This enzyme is proved to be a homodimer in the active form [20]. It would be interesting to see more details in the structures of these two different forms since they might reveal packing differences among the enzyme molecules.

The preliminary structures of these two complexes are being solved, using molecular replacement and direct refinement. From these structures, we found that the electronic density maps of the ligands at the binding site are weaker than those found in other binary or active ternary complexes. This could be explained by the low binding affinity between the inhibitor and the enzyme, which may lead to a low occupancy rate of the ligand in the structure.

The preliminary results also revealed that there may be some changes of conformation of the residues situated in the vicinity of the binding packet. Further explanations will be given once the structures are fully refined.

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