Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of a recombinant Fab fragment in complex with 17β-oestradiol

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Received 19 June 2000
Accepted 26 September 2000

The recombinant Fab fragment of the anti-17β-oestradiol antibody 57-2 has been a target for several protein-engineering experiments. A method for production, purification and crystallization of the Fab fragment alone (apo form) and in complex with the major female sex hormone 17β-oestradiol is reported here. Diffracting apo-form crystals were only obtained with microseeding; crystals of the Fab–steroid complex were produced by co-crystallization in the presence of oestradiol and cross-seeding with the apo-form crystals. The crystals were grown using vapour-diffusion methods with reservoir solutions containing 10–14% PEG 4000 or 8–12% PEG 8000 and Tris–HCl buffer at high pH (9.0–9.5). Both the apo and complex crystals belong to space group $P2_12_12_1$ and diffract to 2.0 Å resolution. High-resolution X-ray data sets suitable for structure determination were collected from flash-cooled crystals using 25% glycerol as the cryoprotectant.

1. Introduction

17β-oestradiol (E2) is one of the most important female steroidal sex hormones, with numerous physiological functions, and is a pivotal clinical analyte. Immunoassays are used to obtain fast and accurate serum E2 levels in a routine manner. However, generation of monoclonal antibodies that have a sufficiently high affinity and specificity for steroid hormones has proven to be very difficult using the conventional hybridoma techniques. The polyclonal antibodies utilized in the current E2 measurement technology have several disadvantages compared with monoclonal antibodies. The most severe problem is a large batch-to-batch variation; the E2 assays therefore require a laborious optimization procedure with each new batch of polyclonal immunoglobulins. Hence, a monoclonal recombinant high-affinity/specificity E2 antibody would be very desirable. Because the E2 concentration in serum can be below 50 pmol l^{-1}, the antibodies should have high (subnanomolar) binding affinities for E2 and low cross-reactivities with other related steroids in the circulation.

We have recently cloned and started to modify and improve a murine anti-oestradiol monoclonal antibody 57-2 that had an original K_a of 5×10^8 for oestradiol and a promising specificity profile, except for having a high cross-reactivity with testosterone [TES; $K_a(\mathrm{TES})/K_a(\mathrm{E2}) \simeq 37\%$]. The phage display technology was used to screen mutant antibody libraries and a number of improved E2 binders were identified (Saviranta *et al.*, 1998; Lamminmäki *et al.*, 1999). The E2-binding characteristics of the modified Fab fragments approach those needed in clinical work. Preliminary serum E2 assays suggest, however, that further engineering of the Fab fragments may still be necessary (U. Lamminmäki, unpublished observations).

Previously, we used molecular modelling to understand how oestradiol binds to the original 57-2 antibody (Lamminmäki *et al.*, 1997). The structural information obtained from displaying homologous sequences on X-ray structures is of a descriptive nature, however, and solid detailed high-resolution information of the oestradiol–Fab interactions is lacking. Currently, there are few steroid–antibody complex crystal structures available in the Protein Data Bank (PDB; Berman *et al.*, 2000): a progesterone–Fab structure (Arevalo *et al.*, 1993), two digoxin–Fab structures (Jeffrey *et al.*, 1993, 1995) and crystal structures of an Fv fragment in complex with oestrone-2-glucuronide and oestriol-3-glucuronide (Trinh *et al.*, 1997). The engineering of further higher affinity mutants and a thorough understanding of the affinity-improving modifications is hampered because of the lack of a high-resolution X-ray structure of the Fab–E2 complex.

In this work, we describe the production, purification, crystallization and preliminary high-resolution X-ray analysis of the Fab fragment of the wild-type 57-2 antibody (Pajunen *et al.*, 1997) alone and in complex with oestradiol. Crystals of the complex could only be produced by co-crystallization in the presence of oestradiol and cross-seeding with

the apo-form crystals. The crystals diffract to 2.0 Å resolution and are suitable for X-ray structure determination.

2. Materials and methods

2.1. Protein production and purification

The Fab fragment was overexpressed in the periplasma of the *Escherichia coli* strain XL-1 in a 4 l batch fermentation. SB media supplemented with 0.2% glucose was used as the nutrition source and ampicillin (10 µ*M*) was used to maintain selection pressure. As an expression vector we used the pCOMB3 plasmid (Barbas *et al.*, 1991). Recombinant protein expression was induced with 100 µ*M* IPTG when the cell-density level reached an OD_{600nm} of 4.0 and growth was continued for 5.5 h after induction. Cells were harvested by centrifugation (6000*g*, 20 min, 277 K) and were stored as pellets at 203 K. The frozen pellets were then defrosted in a warm water bath and the protein was released from the periplasma by osmotic shock by suspending the cells in 20 m*M* Tris–HCl pH 9.0, 2 m*M* EDTA (15 ml buffer per gram of cells) and the debris was removed by centrifugation (10 000*g*, 15 min, 277 K). To precipitate DNA, 0.01% polyethyleneimine (Sigma) was added to the supernatant, followed by centrifugation (as above) after which the pH was adjusted to 5.8 with diluted acetic acid. Purification of the protein was performed in three steps. First, the Fab fragment was concentrated and fractioned with a Streamline (Pharmacia) SP cation-exchange column (75 ml matrix) using 20 m*M* MES pH 6.0 as a running buffer and 50 m*M* MES pH 6.0, 250 m*M* NaCl as the elution buffer. In the second step, solid $(NH_4)_2SO_4$ was added to the eluent [1 *M* final $(NH_4)_2SO_4$ concentration] and the sample was absorbed onto a 20 ml hydrophobic interaction chromatography (HIC) column (HP Sepharose, Pharmacia) that had been equilibrated with 50 m*M* MES pH 6.0, 1 *M* $(NH_4)_2SO_4$, 0.5 m*M* EDTA. The protein was eluted from the column with a decreasing $(NH_4)_2SO_4$ concentration gradient and the Fab-containing fractions were pooled and diluted with deionized H_2O to lower the conductivity to below 3.0 mS. The final step was performed with a Mono S (10/10) cation-exchange column (Pharmacia) using 50 m*M* MES pH 6.0 as the equilibration and running buffer and a NaCl gradient for elution. The appropriate fractions were pooled and the buffer was exchanged to 10 m*M* MES pH 6.0 using Centricon 30 concentrators (Amicon) and a NAP-10 (Pharmacia) desalting column. The purity of the protein was confirmed with SDS–PAGE gels.

2.2. Crystallization

Sparse-matrix screens (Hampton Crystal Screen Kits 1 and 2), various different polyethyleneglycol molecules (M_r 1000–20 000), a wide range of pHs and different temperatures were systematically screened using the hanging-drop and sitting-drop vapour-diffusion methods. Crystals were obtained after 1 y in the incubator at 277 K with 5% PEG 8000, 100 m*M* Tris–HCl pH 8.7 and a protein concentration of 30 mg ml^{-1}. The initial crystals were thin and fragile stacked plates and were unsuitable for diffraction studies. Diffracting crystals were obtained with microseeding: (i) the fragile plates were crushed in the crystallization drops, (ii) the seeds were diluted 1:2000–1:4000 with the reservoir solution and (iii) were then moved with a cat's whisker to a fresh protein-containing sitting drop that had been equilibrated for 2 d at 277 K. The pre-equilibrated drops contained 3 µl protein solution (15–25 mg ml^{-1}) mixed with 3 µl of reservoir solution consisting of 50 m*M* Tris–HCl pH 9.0–9.5, 8–12% PEG 8000 or 10–14% PEG 4000. The target drops were quickly streaked, sealed and stored at 277 K. To obtain crystals of the complex, the hydrophobic 17-β-oestradiol (0.1–1 m*M*; Sigma) was dissolved in reservoir solution supplemented with 5–20% ethanol and was then added to the drops containing the fully grown crystals. In co-crystallization trials with E2, the microseeding procedure (above) was used but 1 µl of steroid-containing solution (12% PEG 8000, 50 m*M* Tris–HCl pH 9.1, 20% EtOH, 1 or 5 m*M* oestradiol) was added into the drops in the pre-equilibration step and the amount of reservoir solution was decreased from 3 to 2 µl.

2.3. Cryocooling, data collection and molecular replacement

The crystals were cooled by transferring and soaking them (1 min per solution) through a series of five solutions containing increasing (5, 10, 15, 20, 25%) amounts of glycerol in 12% PEG 8000, 50 m*M* Tris–HCl pH 9.1; the crystals were then picked from the cryoprotectant solution into fibre loops (Hampton) and flash-frozen in a nitrogen-gas stream at 100 K. The X-ray data was collected at 100 K using Cu $K\alpha$ radiation from a Rigaku RU-200HB rotating-anode X-ray generator (50 kV and 180 mA) equipped with an R-AXIS IIC image-plate detector and a graphite monochromator. The data sets were collected from single crystals and were indexed, integrated and scaled with the programs *DENZO* and *SCALEPACK* (Otwinowski, 1993). The program *CNS* (Brunger *et al.*, 1998) was used in the Matthews coefficient, electron-density map and molecular-replacement (MR) calculations. 7–10% of the integrated reflections were set aside and were only used for evaluating R_{free} (Brünger, 1992). A hybrid Fab-fragment model that was used in the MR studies was constructed from the PDB files 1tet (variable heavy chain; Shoham, 1993) and 1fdl (variable light chain and both constant domains; Fischmann *et al.*, 1991); the electron-density maps were inspected with the program *O* (Jones *et al.*, 1991).

3. Results and discussion

In the purification procedure, the protein G affinity chromatography that is conventionally utilized for Fab purification was not used in order to avoid the potentially harmful exposure of the protein to low pH. The Fab bound tightly to the HIC column and this purification step was very effective. The small hydrophobic ligands of the HIC matrix might interact directly with the abundant non-polar amino acids in the combining site of the Fab 57-2 (Lamminmäki *et al.*, 1997), suggesting that HIC chromatography might be a very useful way to purify other antibodies recognizing hydrophobic ligands. A typical yield of pure protein from a production/purification batch was around 20 mg.

Sparse-matrix screens (Jancarik & Kim, 1991) commonly used in initial crystallization experiments failed to produce any crystals. The usefulness of PEGs in the crystallization of antibody fragments has been demonstrated in many experiments. We obtained crystals with two different high molecular-weight PEGs (4000 and 8000). The trials with PEG 3350, although previously reported to be very successful with another steroid-binding Fab fragment (Valjakka *et al.*, 2000), did not produce any crystals in our hands. The fact that diffracting crystals of the Fab 57-2 grew at high pH (>9.0) points out the need to use wide pH ranges in the PEG screens. The initial crystals obtained from PEG/pH screens were too fragile for data collection and numerous trials to refine the conditions failed. The production of diffracting crystals was difficult; however, using the described

Table 1
Diffraction and crystal data of the recombinant Fab fragment of anti-oestradiol antibody 57-2.

Values for the highest resolution shells (2.59–2.5 Å in the apo form and 2.25–2.15 Å in the complex) are shown in parentheses.

Crystal	Apo	Steroid–Fab complex
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 48.3	a = 48.2
	b = 64.5	b = 64.5
	c = 165.7	c = 165.3
Measured reflections	166126	131703
Unique	17281	25614
Resolution (Å)	50–2.5	50–2.15
R_{merge} (%)	5.2 (7.8)	5.1 (13.5)
Multiplicity	4.3 (1.9)	2.7 (1.8)
$I > 3\sigma(I)$ (%)	93.5 (84.4)	81.0 (67.0)
Completeness (%)	93.4 (72.7)	88.7 (71.9)

microseeding procedure we managed to obtain crystals (Fig. 1) suitable for high-resolution structure determination. Brick-shaped crystals appeared in 2–4 d and grew to their maximal size in two to three weeks. Once optimal conditions for the microseeding were found, crystals could be grown reproducibly. The crystals diffract below 2 Å resolution but deteriorate rapidly when exposed to X-rays at room temperature; high-resolution data sets could only be collected with flash-cooled crystals (Table 1). Various cryoprotectants were screened but we could only cool the crystals with a series of glycerol soaks. Only small crystals (largest dimension <0.25 mm) survived the cryo-soaks.

The crystals belong to the primitive orthorhombic lattice type; unit-cell parameters are shown in Table 1. Using 50 kDa as the molecular weight of the Fab fragment, 1.3 g cm^{-3} as the macromolecular density and the unit-cell parameters from Table 1, the calculated Matthews coefficient (Matthews, 1968) was 2.6 Å^3 Da^{-1} and the crystal solvent content was 50.3%. These values are consistent with a single Fab fragment in the asymmetric unit. The space group of the crystal was $P2_12_12_1$ as determined from systematic absences in the diffraction patterns and by processing and scaling the diffraction data in different primitive orthorhombic space groups followed by MR studies (Adams *et al.*, 1999). The value of the Patterson correlation coefficient (Brünger, 1990) of the best MR solution in the correct space group was twice as high as the best values obtained in the incorrect space groups. After a rigid-body refinement followed by a torsion-angle dynamics simulated-annealing stage (Adams *et al.*, 1997), the R_{free} fell to 37% and initial maps (resolution 50–2.7 Å) were calculated. When various electron-density maps were inspected using a graphics workstation, it became obvious that no steroid was bound in the E2-soaked crystals. However, clear E2 electron density [>5σ over the mean density in $(F_{obs} - F_{calc})\exp(i\alpha_{calc})$ maps] was seen in the maps generated with X-ray data collected from crystals obtained with microseeding and co-crystallization in the presence of oestradiol using crushed apo-form crystals as the microseeds.

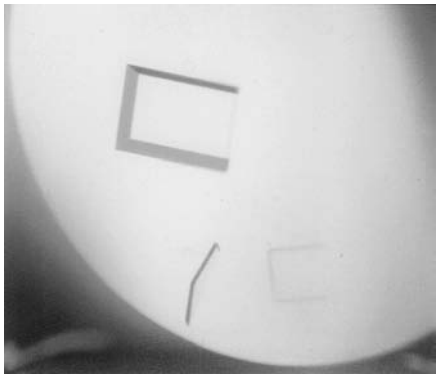

Figure 1
Apo-form crystals of the Fab fragment of anti-oestradiol antibody 57-2 obtained with microseeding. The dimensions of the largest crystal are approximately 0.6 × 0.3 × 0.1 mm.

In this work, we have described in detail how to produce, purify, crystallize and collect high-resolution X-ray data from a 17β-oestradiol–Fab fragment complex. The initial electron-density maps showed that the method to obtain crystals of the E2–Fab complex is not to soak the hydrophobic steroid into the fully grown crystals but to co-crystallize the protein with the steroid. The fact that the steroid–Fab complex crystals could be obtained by cross-seeding with the apo-form microcrystals implies that upon binding of the steroid the structural rearrangements in the protein are minor. This is also in accordance with the very small differences in the unit-cell parameters between the apo and complex crystals (Table 1). We are currently refining the structures and hope to collect even higher resolution X-ray data with the novel confocal optics that are being installed in our laboratory.

The monitoring of steroid levels in blood samples using antibody-based assays is of great clinical value. We believe that detailed high-resolution information from the E2–antibody crystal structures will add to our understanding of the complex nature of the immunoglobulin–steroid interactions and will be the basis for future engineering of anti-oestradiol antibodies with improved properties.

We thank Professors Adrian Goldman and Mark Johnson for the opportunity to use laboratory facilities. This study was supported by the Academy of Finland and Technology Development Centre in Finland (TEKES). JK is a post-doctoral fellow of the Academy of Finland.

References

Adams, P. D., Pannu, N. S., Read, R. J. & Brünger, A. T. (1997). *Proc. Natl Acad. Sci. USA*, **94**, 5018–5023.
Adams, P. D., Pannu, N. S., Read, R. J. & Brunger, A. T. (1999). *Acta Cryst.* D**55**, 181–190.
Arevalo, J. H., Taussig, M. J. & Wilson, I. A. (1993). *Nature (London)*, **365**, 859–863.
Barbas, C. F. III, Kang, A. S., Lerner, R. A. & Benkovic, S. J. (1991). *Proc. Natl Acad. Sci. USA*, **88**, 7978–7982.
Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. & Bourne, P. E. (2000). *Nucleic Acids Res.* **28**, 235–242.
Brünger, A. T. (1990). *Acta Cryst.* A**46**, 46–57.
Brünger, A. T. (1992). *Nature (London)*, **355**, 472–475.
Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.
Fischmann, T. O., Bentley, G. A., Bhat, T. N., Boulot, G., Mariuzza, R. A., Phillips, S. E., Tello, D. & Poljak, R. J. (1991). *J. Biol. Chem.* **266**, 12915–12920.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Jeffrey, P. D., Schildbach, J. F., Chang, C. Y., Kussie, P. H., Margolies, M. N. & Sheriff, S. (1995). *J. Mol. Biol.* **248**, 344–360.
Jeffrey, P. D., Strong, R. K., Sieker, L. C., Chang, C. Y., Campbell, R. L., Petsko, G. A., Haber, E., Margolies, M. N. & Sheriff, S. (1993). *Proc. Natl Acad. Sci. USA*, **90**, 10310–10314.
Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991). *Acta Cryst.* A**47**, 110–119.
Lamminmäki, U., Pauperio, S., Westerlund-Karlsson, A., Karvinen, J., Virtanen, P. L., Lovgren, T. & Saviranta, P. (1999). *J. Mol. Biol.* **291**, 589–602.
Lamminmäki, U., Villoutreix, B. O., Jauria, P., Saviranta, P., Vihinen, M., Nilsson, L., Teleman, O. & Lovgren, T. (1997). *Mol. Immunol.* **34**, 1215–1226.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. (1993). *DENZO and SCALEPACK. Data Processing and Scaling Programs.* Yale University, New Haven, Connecticut, USA.
Pajunen, M., Saviranta, P., Jauria, P., Karp, M., Pettersson, K., Mantsala, P. & Lovgren, T. (1997). *Biochim. Biophys. Acta*, **1351**, 192–202.
Saviranta, P., Pajunen, M., Jauria, P., Karp, M., Pettersson, K., Mantsala, P. & Lovgren, T. (1998). *Protein Eng.* **11**, 143–152.
Shoham, M. (1993). *J. Mol. Biol.* **232**, 1169–1175.
Trinh, C. H., Hemmington, S. D., Verhoeyen, M. E. & Phillips, S. E. (1997). *Structure*, **5**, 937–948.
Valjakka, J., Hemminki, A., Teerinen, T., Takkinen, K. & Rouvinen, J. (2000). *Acta Cryst.* D**56**, 218–221.