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X-ray studies of recombinant anti-testosterone Fab fragments: the use of PEG 3350 in crystallization

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Recombinant anti-testosterone wild-type Fab fragment and mutant Fab fragments with high binding selectivity developed by protein engineering have been crystallized with and without ligands. Crystals of these Fab fragments were obtained by the vapour-diffusion technique at room temperature using solutions of PEG 3350 with various biological buffers and with a wide pH range. So far, five data sets have been collected from crystals of three Fab–antigen complexes and from two uncomplexed Fab fragments, with resolutions ranging from 2.10 to 3.1 Å. Crystallization conditions for Fab fragments were found by using modifications of the low ionic strength PEG 3350 series. Suitable concentrations of PEG 400, MPD and glycerol solutions for use as cryoprotectants in PEG 3350 solutions have been determined. One useful observation was that PEG 3350 is able to work alone as a cryoprotectant. The screening protocol used requires a smaller amount of protein material to achieve auspicious pre-crystals than previously. Results support the claim that PEG 3350 is more suitable for the crystallization of Fab fragments than higher molecular weight PEGs.

1. Introduction

The highly specific high-affinity interaction of an antibody with an antigen forms the basis of the immunological techniques widely used in diagnostic, medical and research applications. Nonetheless, there are analytes, *e.g.* steroid hormones, for which the generation of monoclonal antibodies with sufficiently high affinity combined with high specificity using conventional hybridoma technology has been extremely difficult or impossible. The closely related steroid structures and the significant variations in their relative concentrations even between normal healthy individuals demand an extremely high selectivity of the antibodies intended for use in clinical immunoassays. Random mutagenesis of the complementarity-determining regions (CDR) and phage display selection have been successfully used to tailor the binding site of a monoclonal anti-testosterone antibody (Hemminki, Niemi, Hautoniemi *et al.*, 1998; Hemminki, Niemi, Hoffren *et al.*, 1998; Hemminki *et al.*, 2000). When compared with the wild type, the best mutant Fab fragment (FAB77) has an approximately 40-fold increase in affinity ($K_d = 3 \times 10^{-10}\ M$) and a 50-fold lower cross-reactivity (0.006%) to the most problematic cross-reacting steroid, dehydroepiandrosterone sulfate (DHEAS; Hemminki *et al.*, 2000). The mutant FAB77 works accurately in an immunoassay of clinical samples and correlates well with a commercial reference immunoassay. In order to understand the mechanism of this high affinity and specificity binding mode, the wild-type (FabWT) and mutant Fab fragments (FAB77, FAB60) have been subjected to crystallization experiments.

To date, structural information on steroid–protein interactions has been based on the structures of free steroids (Duax & Norton, 1975; Griffin *et al.*, 1984) and on crystallographic analyses of the unliganded forms of steroid-binding proteins (Bally & Delettre, 1989; Vrielink *et al.*, 1991; Ghosh *et al.*, 1991). A very good model, describing and resolving the interactions between steroids and proteins and the basis of binding selectivity, is the complex structure of monoclonal anti-progesterone antibody DB3 with progesterone and its derivatives (Arevalo *et al.*, 1993). This complex structure has also been used to calculate a model for the interactions between testosterone and the anti-testosterone wild-type Fab fragment (Hemminki, Niemi, Hoffren *et al.*, 1998). However, the most accurate way to identify interactions of an antibody and its hapten is to solve the complex crystal structure. A number of Fab structures have been determined, but there have been difficulties in obtaining crystals that diffract at high resolution.

Insights into the different types of binding interactions provide good opportunities for improving the hapten-binding properties.

Table 1
Preliminary crystallization conditions and cryoprotectants using PEG 3350 as a precipitant.

	Crystallization solution										Cryoprotectant (%)			
PEG 3350 (%)	Citric acid pH 3.0	NaAc pH 4.0	Citrate pH 4.5	BTP pH 5.5	MES pH 6.0	ADA pH 6.5	Cacolydate pH 7.0	HEPES pH 7.5	Tris pH 8.0	Bicine pH 8.5	PEG 400	MPD	Glycerol	PEG 3350
5	1	2	3	4	5	6	7	8	9	10	>12	>14	22.5	35
10	11	12	13	14	15	16	17	18	19	20	>12	>12	22.5	35
15	21	22	23	24	25	26	27	28	29	30	>12	>12	17.5	35
20	31	32	33	34	35	36	37	38	39	40	>12	>10	15	35

Independent structures of the native Fab fragment and its complexes would show both the details of the interactions between the antibody and the hapten, and whether the hapten binding induces conformational changes in the antibody. In recent years, several structures have been reported for Fab–steroid complexes (Arevalo *et al.*, 1993, 1994). Diffraction limits around 3 Å have usually been reported in the literature; in only a few cases were resolutions of 2 Å or better obtained. Usually, the antibody or the proteolytic or recombinant antibody fragment crystals have been obtained with ammonium sulfate or PEG 8000 (polyethylene glycol) as the precipitant. Harris *et al.* (1995) were the first to report the crystallization of monoclonal antibodies at low ionic strength with PEG 3350 solution; these low ionic strength conditions for crystallization have proved to be effective for determining the preliminary crystallization conditions of intact monoclonal antibodies as well as their fragments.

In this paper, we describe the elucidation of crystallization conditions and preliminary X-ray studies of the recombinant anti-testosterone Fab fragments and their complexes with testosterone. The crystallization conditions were found by using only ten individual buffers at different pH unit steps of the low ionic strength PEG 3350 series. The advantage of this established crystallization experiment is that it requires less protein material for generation of auspicious pre-crystals. We also present the results of cryocooling experiments in PEG 3350 with common cryoprotectants.

2. Materials and methods

2.1. Crystallization

The wild-type FabWT and the mutant Fab fragments FAB77 and FAB60 were produced as soluble proteins in high cell density *Escherichia coli* bioreactor cultivations and purified first by a cation-exchange column and then by a protein G column, as described in Hemminki *et al.* (2000). The crystallization conditions were tested by the hanging-drop vapour-diffusion method in VDX 24-well tissue-culture plates. The crystallization conditions were screened for native Fab fragments and testosterone and DHEAS complexes. The crystal screen that was used as a starting point in every case was produced using the procedure outlined below.

The concentration of high-purity monodisperse PEG 3350 (Hampton Research) solutions varied from 5 to 20%. The pH of the PEG 3350 solutions was in the range 3–8.5. Every PEG 3350 solution contains one of the following buffers: citric acid (pH 3.0), sodium acetate (pH 4.0), sodium citrate (pH 5.0), Bis–Tris propane (pH 5.5), MES (pH 6.0), ADA (pH 6.5), cacodylate (pH 7.0), HEPES (pH 7.5), TRIS (pH 8.0) or Bicine (pH 8.5), supplied by the Sigma Chemical Company. The concentration of all buffers were 0.1 *M*. The pH of each buffer was adjusted before it was added to the PEG 3350 solution. The hanging drops typically consisted of 2 µl of protein solution in the same volume of reservoir solution and an equal volume of testosterone or DHEAS solution. The individual wells had been pre-filled with 0.5 ml of precipitating solution. All hanging drops had a protein concentration of 4–20 mg ml^{-1} with or without saturated steroid dissolved in ethanol. The drops were allowed to equilibrate at a constant temperature of 293 K. Crystals normally appeared within a few days and continued to appear for several weeks.

2.2. Cryocooling

Cryoprotectants are compounds that prevent ice formation on cooling when added to aqueous solutions. Typically, the cryoprotectant is added to a portion of mother liquor, usually in the range 10–35% by volume (Rodgers, 1994). Cryo-trials in this study were carried out on a microscale with concentrations of PEG 400, MPD and glycerol solutions in the range 10–40% in PEG 3350 solution (5–20%) (Table 1). In all cases, we have found that flipping the crystal into the cold nitrogen stream was not quick enough and, therefore, the mosaicity after freezing usually increased. To avoid this problem, we used liquid nitrogen for crystal plunging. The MPD concentration required for cryoprotection was at least 12% when the concentration of PEG 3350 was at least 10%. The usable concentration for PEG 400 was over 12% for all solutions of PEG 3350. When glycerol was used for cryoprotection, the concentration had to be increased to greater than 22.5% in order to prevent the formation of ice rings. PEG 3350 was an intrinsic cryoprotectant liquid when its concentration was 35%.

2.3. Data collection

Data collection utilized a Rigaku RU-200HB rotating-anode generator (operated at 50 kV and 100 mA) equipped with a graphite monochromator and an R-AXIS IIC imaging-plate area detector (the crystal-to-detector distance was 120 mm). Later, a new MSC Confocal Blue Optics (Yang *et al.*, 1999) was installed on our X-ray source. Data sets for native wild-type and mutant FAB77 were collected with this system. For data collection, crystals were harvested into a solution of cryoprotectant and then mounted in nylon loops and flash-frozen in a cold stream at 120 K (using an Oxford Cryosystems Cryostream cooler). Data were collected using 1–1.5° oscillations; 180° were collected in total. The exposure time was 30 min when using the graphite monochromator and 10 min when using the Confocal Optics. Unfortunately, we were unable to collect data from the wild-type Fab complexed with testosterone and from the mutant fragment FAB60 at 120 K. This data was measured at room temperature in a 0.7 mm capillary. The data sets were processed using the *DENZO* and *SCALEPACK* programs (Otwinowski, 1993) and the true space groups were deduced with the aid of the *XPREP* program (*SHELXTL* software package).

Table 2
Crystal and diffraction data for recombinant anti-testosterone Fab fragments.

Protein	Wild type	Wild type	Mutant FAB60	Mutant FAB77	Mutant FAB77
Ligand	—	Testosterone	Testosterone	—	Testosterone
Drop volume (µl)	4	6	6	4	6
Buffer	0.1 *M* BTP	0.1 *M* BTP	0.1 *M* MES	0.1 *M* MES	0.1 *M* MES
pH	5.60	5.6	7.5	5.8	6.0
Precipitant (*w*/*v*)	15% PEG 3350	15% PEG 3350	13% PEG 3350	16% PEG 3350	15% PEG 3350
Space group	*I*2224	*I*222	$P2_12_12_1$	$C222_1$	$P6_2$
Unit-cell dimensions (Å)	*a* = 90.46	*a* = 90.82	*a* = 52.78	*a* = 71.71	*a* = 82.25
	b = 90.98	*b* = 94.91	*b* = 81.56	*b* = 88.53,	*b* = 82.25
	c = 137.10	*c* = 137.92	*c* = 97.31	*c* = 143.94	*c* = 120.06
Mosaicity (°)	0.954	0.506	0.257	0.755	0.354
Temperature (K)	120	293	293	120	120
Cryoprotectant	35% PEG 3350	—	—	35% PEG 3350	20% MPD
Measured reflections	103739	62144	23437	120393	88701
Unique reflections	15557	12124	7973	25386	24335
Resolution (Å)	2.72	2.65	3.1	2.1	2.15
R_{merge} (%)	6.9 (30.4, 2.72–2.82 Å shell)	6.6 (31.9, 2.65–2.74 Å shell)	10.0 (26.9, 3.1–3.21 Å shell)	7.0 (32.6, 2.18–2.1 Å shell)	5.7 (33.0, 2.15–2.23 Å shell)
Completeness (%)	99.9 (99.9, 2.72–2.82 Å shell)	68.5 (73.3, 2.65–2.74 Å shell)	98.7 (99.2, 3.1–3.21 Å shell)	93.6 (87.9, 2.10–2.18 Å shell)	97.0 (93.3, 2.15–2.23 Å shell)

3. Results

3.1. Crystallization

3.1.1. Crystallization of the native Fab fragments. The uncomplexed anti-testosterone Fab fragments were crystallized using a modified crystal screen PEG 3350 series for antibodies (Table 1). Crystals were obtained using equal volumes of a 10–20 mg ml^{-1} protein solution and a precipitant solution containing 15% PEG 3350 for the mutant FAB77 and for the FabWT. Pre-crystals appeared in solutions 21, 22, 23, 24, 25 and 26; in solutions 21 and 26 there were only FAB77 pre-crystals. The best crystals for the X-ray study were grown in solution 25 for the FAB77 mutant and in solution 24 for FabWT. Pre-crystals for FAB60 appeared in solutions 21, 26, 36, 39 and 40. All crystallization details, data collection and crystal parameters are shown in Table 2.

3.1.2. Co-crystallization of the Fab fragments with testosterone. Crystals of the FAB77 fragment were obtained by the vapour-diffusion technique using solutions of PEG 3350 with various biological buffers (Table 1) over a wide pH range (3.5–8). The present series gave crystals from solutions 21, 24, 25, 26, 27, 28, 29 and 30. The best crystals for X-ray experiments could be grown using equal volumes of 10–20 mg ml^{-1} protein solution and a precipitant solution containing 15% PEG 3350 buffered at pH 6.0 with 0.1 *M* MES (solution 25). When measured, this crystal diffracted to a resolution of 2.15 Å. Data were also measured from crystals of FAB60 and FabWT; these crystallized in solutions 28 and 24, respectively. Other pre-crystals were obtained in solutions 30 and 40 for FabWT. FAB60 was found in solutions 23, 24, 25 and 35. All information on the data collection and crystal parameters is shown in Table 2.

3.1.3. Co-crystallization of the Fab fragments with DHEAS. The Fab fragment with DHEAS crystallized in the same pre-crystallization conditions as those described above. The pre-crystals appeared in 20% PEG 3350 solutions 33, 35, 36, 37 and 38 for FabWT and FAB60 precrystals appeared in 15% PEG 3350 solutions 25, 26, 27 and 28. Unfortunately, these crystals were all badly disordered. Consequently, more accurate conditions are still being sought.

3.1.4. Crystallization of the FAB77 fragment with PEG 8000. PEG 8000 has been a successful precipitant for the ordinary crystallization of Fab fragments. We have also tested the use of PEG 8000 in the crystallization of FAB77. The FAB77 native and complexed fragments crystallized from solutions containing 10–20% PEG 8000, 50 m*M* K_2HPO_3 and 50 m*M* acetate buffer at pH 4.5. All crystals grown from this solution diffracted at only around 4 Å. For the native FAB77 crystals, however, diffraction was observed to 3.5 Å.

3.2. X-ray data collection

In total, five data sets from the wild-type Fab fragment and mutant Fab fragments with or without testosterone were collected. Despite the high similarity between the crystallization of the wild-type and mutant Fabs, we obtained four crystal forms. The wild-type Fab crystallized in space group *I*222 with and without testosterone. However, the testosterone had a clear effect in the crystallization of FAB77. The native FAB77 crystallized in space group $C222_1$, but the testosterone complex crystallized in space group $P6_2$. The fourth crystal form $P2_12_12_1$ was obtained from the mutant FAB60.

Low temperature was a clear prerequisite for the collection of higher resolution data. We regularly found strong crystal decay when exposing Fab crystals. Even large crystals decomposed quickly and the collection of a complete higher resolution data set was not possible. This is the reason for the low completeness of the wild-type complex data set and the low resolution of the mutant FAB60 data set. Much better results were obtained from cryocooled crystals. The resolution was significantly higher and we were able to collect almost complete data sets. New Confocal X-ray Optics allowed us to collect data sets much more rapidly and we obtained higher multiplicity. We also were able to improve the resolution of native wild type from 2.9 to 2.7 Å. A summary and comparison of the X-ray diffraction data for all measured Fab fragments is given in Table 2.

4. Discussion

All the PEGs in the molecular-weight range 400–20 000 Da have been used in protein crystallization, but with Fab fragments the PEG of molecular weight 8000 Da has usually been used. Since the successful antibody crystallization experiments by Harris *et al.* (1995), PEG 3350 has often been used for Fab fragments. The molecular size of PEG 3350 might be more suitable because it is a smaller molecule than, for example, PEG 8000 and thus can probably enter the crystals and set up favourable contacts between interior molecules (McPherson, 1999). In X-ray studies, however, flexible macromolecules, in particular those with multiple domains such as immunoglobulins, have frequently been observed to display some inherent disorder. The crystals of such molecules generally diffract less than those of immobile stable macromolecules. The inability of many dynamic proteins to crystallize is a consequence of a conformational or other heterogeneous disorder. Crystals of macromolecular complexes represent even more extreme situations. Elements of the complexes may occasionally be missing and there may be multiple modes of association (McPherson *et al.*, 1996). Because the complete antibodies are massive and mobile, in ordinary studies the antigen-binding Fab fragments only have been used instead of the entire antibody.

In conclusion, the crystallization conditions presented here were proved to be effective for testosterone-specific mono-

clonal antibody Fab fragments. Both testosterone-complexed Fab fragments and native Fab fragments were crystallized using PEG 3350 as precipitant, using a protein material consumption of less than 100 µl. Furthermore, our results indicate that the FabWT and mutant FAB77 crystallize more efficiently with testosterone, most probably because of an increase of rigidity in the loop movements upon ligand binding. This effect is probably caused because of a reduction in variable loop movements when testosterone binds to Fab fragments. In addition, difficulties in obtaining co-crystals with DHEAS may be caused by different binding or the induction by DHEAS of unspecified disorders in the crystal contact area. Despite the fact that we have been successful in obtaining a high-resolution data set for FAB77 fragment with testosterone, it is still difficult to stabilize the crystals during cryocooling. Nonetheless, we suppose that PEG 3350 would be a good choice for preliminary screening of Fab fragments, especially if only a small amount of protein material is available. PEG 3350 solutions on their own are good cryoprotectants when the concentration of the PEG is at least 35%. This will make the choice of PEG 3350 even more favourable for the crystallization of Fabs.

We have solved the three-dimensional structure of the mutant FAB77 fragment with testosterone by the molecular-replacement method. The other data sets measured (native FAB77 and FabWT, FAB60 complex) are being prepared in order to solve the three-dimensional structures. Knowledge of the different complex structures of these anti-testosterone Fab fragments will help us to determine the important residues for testosterone binding and probably to deduce those residues that should be targeted for mutagenesis to achieve even higher binding selectivity.

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