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Crystallization of an intact GST-estrogen receptor hormone binding domain fusion protein

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Abstract

Crystals of an intact GST–estrogen receptor hormone binding domain fusion protein have been grown from solutions of MPD. The crystals grew as clusters of thin plates and needles of maximum dimensions 100 × 20 × 1 μm but were unsuitable for X-ray diffraction analysis. However, examination by electron microscopy shows an ordered lattice in which the protein molecules are clearly visible. Image analysis of electron micrographs of the protein crystals revealed electron stain-excluding density which showed a two-domain trimeric structure in projection, with each molecule of dimensions 12.0 × 5.0 nm diameter. The use of GST-fusion proteins in crystallization are discussed.

1. Abbreviations

GST, glutathione-*s*-transferase; MPD 2-methyl-2-4 pentanediol; EDTA, ethylene diamine tetraacetic acid; DTT, dithiothreitol; IPTG, isopropyl-1-thio-β-D-galactopyranoside; RXR, retinoid-X receptor; ERHBD, estrogen receptor hormone binding domain.

2. Introduction

Many recombinant proteins are now being expressed using a variety of expression vectors which allow the incorporation of an affinity tag to facilitate purification. These include for example, the addition of a polyhistidine sequence to the protein of interest, which can be used in immobilized metal chelation chromatography allowing one-step purification schemes (Smith *et al.*, 1988). Other types of systems fuse the protein of interest to other proteins such as GST or maltose-binding protein, both of which have high-affinity ligand specificities which can be immobilized on columns suitable for chromatography (Smith & Johnson, 1988; Riggs, 1992). For crystallographic studies these tags or fusion proteins are usually removed enzymatically, by engineering a specific protease site into the protein, for example, thrombin or factor X (Skelley & Madden, 1996). Recently, it has been suggested that fusion proteins could be of use in helping to solubilize or stabilize proteins which are otherwise difficult to crystallize (Lim *et al.*, 1994; Carter *et al.*, 1994). It has also been suggested that GST-fusion proteins could be used to obtain phase information in molecular replacement studies as the structure of the GST moeity is known (McTigue *et al.*, 1995). However, as far as we know, there has been only one report of a GST-fusion protein crystallization, this being a small (six amino-acid residues) peptide antigen (Lim *et al.*, 1994). A similar process has used lysozyme as a carrier protein to crystallize a 14-residue peptide from the human fibrinogen γ chain (Donahue *et al.*, 1994). Other methods used to overcome difficult crystallization problems, include co-crystallization of the protein of interest with co-factors (Zhu *et al.*, 1994), use of Fab complexes with the target protein (Iwata *et al.*, 1995), or proteolytic cleavage (Sanderson *et al.*, 1990). There are several reported examples of proteins being crystallized with an intact polyhistidine tag (Bourget *et al.*, 1995).

The hormone-binding domain of the estrogen receptor is responsible for 17-β estradiol ligand binding and protein dimerization leading to transcriptional activation. Prevention of dimerization by anti-estrogens is a potential therapeutic target in the treatment of estrogen-dependent breast cancers (Arbuckle *et al.*, 1992). Structural studies of the GST-estrogen receptor hormone binding domain were initiated, to elucidate the effect of 17-β estradiol and anti-estrogens, such as tamoxifen on conformation of the receptor. Size-exclusion chromatography shows that the fusion protein is dimeric when unliganded, and in the presence of estradiol, but is monomeric in the presence of the anti-estrogen (Arbuckle, 1994). Here we report the crystallization of the hormone-binding domain of the mouse estrogen receptor as a GST-fusion protein.

3. Materials and methods

The mouse estrogen receptor hormone-binding domain (ERHBD; residues 281–599) was cloned as a GST-fusion protein into the pGEX-1 lambda expression system, and expressed in *E. coli* (BL21) cells. The cells (400 ml) were cultured to an optical density of 0.8 at 600 nm, and protein expression induced by the addition of IPTG (1 m*M*). Cells were incubated for a further 4 h before harvesting by centrifugation at 4000 rev min^{-1} for 20 min in a Beckman J6 centrifuge. Pellets were either used immediately or stored frozen at 203 K. Cell pellets were suspended in 45 ml of extraction buffer, Phosphate-buffered saline, 10%(*v*/*v*) glycerol, 1 m*M* EDTA, 10 m*M* DTT, 1%(*v*/*v*) Triton-X 100, 0.02%(*w*/*v*) sodium azide, 1 mg ml^{-1} lysozyme, plus protease inhibitors and adjusted to pH 7.4. After sonication for 1 min at maximum power the extract was clarified by centrifugation at 40 000 rev min^{-1} for 1 h at 277 K, in a Beckman 50.2Ti rotor. The supernatant was removed and stored at 277 K and the pellets re-sonicated with 35 ml of extraction buffer as described above. After ultracentrifugation the supernatants were pooled.

Supernatants extracted from pellets representing approximately 6 l of original cell culture, were applied at a flow rate

of 2 ml min^{-1} to a 2.6 × 2 cm column of glutathione-coupled Sepharose, previously equilibrated with PBS, 10%(*v*/*v*) glycerol, 1%(*v*/*v*) Tween, 0.02%(*w*/*v*) sodium azide, pH 7.4. The column was washed with a further 30 ml of the same buffer, followed by 30 ml of 20 m*M* Hepes, 0.02%(*w*/*v*) sodium azide pH 8.0. The GST–ERHBD, along with free GST was eluted by the same buffer containing 20 m*M* reduced glutathione. The eluate was adjusted to 0.05%(*w*/*v*) *β*-octylglucoside, and concentrated to 5 mg ml^{-1}. The fusion protein was further purified by passage down a Superose 12 size-exclusion column equilibrated in 20 m*M* Hepes, 0.2 *M* NaCl, 0.05%(*w*/*v*) *β*-octylglucoside pH 8.0, to remove free GST. The final eluate was desalted and dialyzed into 20 m*M* Hepes, 0.02%(*w*/*v*) sodium azide, 0.05%(*w*/*v*) *β*-octylglucoside, pH 8.0, and concentrated to 8 mg ml^{-1} in a Centricon 10 for crystallization trials.

Crystallization trials were carried out by the hanging-drop vapour-diffusion method, using siliconized cover slips and Linbro 24-well tissue-culture trays (McPherson, 1982). Initial screening was carried out using the sparse-matrix screening kits from Hampton Research (Crystal screen HI and HII, Jancarik & Kim, 1991; Cudney *et al.*, 1994) 2 µl of the protein, 8 mg ml^{-1} in 20 m*M* Hepes, 20 m*M* NaCl, 0.05%(*w*/*v*) *β*-octylglucoside, pH 8.0, were mixed with 2 µl of the precipitating solution at 293 K. The composition of the crystals was investigated for protein content by using transmission electron microscopy (EM). Negatively stained specimens for EM were prepared as follows. Suitable microcrystals of GST–ERHBD produced by the hanging-drop method were collected on air-glow-discharged grids and floated on a drop of 1%(*w*/*v*) uranyl acetate for 20 s. Excess stain was blotted and the grid was dried in air prior to examination in a Zeiss EM 10CR transmission electron microscope, operating at an accelerating voltage of 100 kV. Micrographs of negatively stained crystals were taken at 39 000× magnification, and areas suitable for analysis were selected by optical diffraction, grabbed and digitized using a charge-coupled device (CCD) camera attached to a Dell 166 (UK) microcomputer running *Semper* software from Synoptics (Cambridge, UK). Molecular modelling was carried out using the graphics program *PREPI* (S. Islam & M. Sternberg, Imperial Cancer Research Fund) on a Silicon Graphics Indigo 2 workstation.

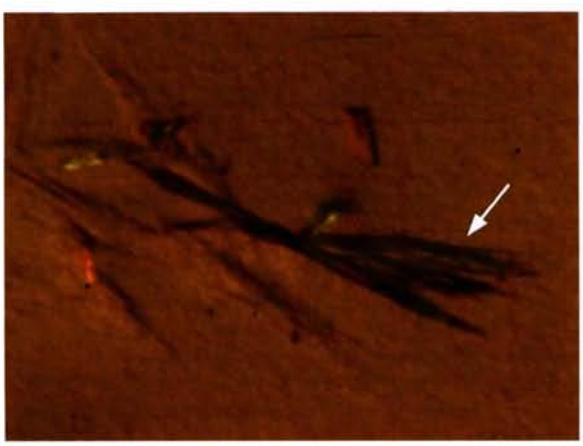

Fig. 1. Crystals of GST–ERHBD fusion protein of dimensions 100 × 20 × 1 µm, indicated by the arrow.

4. Results and discussion

The initial GST–ERHBD crystals grew in condition HI 21, comprising 0.2 *M* magnesium acetate, 0.1 *M* sodium cacodylate, 30%(*v*/*v*) MPD, pH 6.5. The crystals grew as thin plates (<1 µm thick) from a heavy precipitate and took one to two weeks to appear. Optimization of the conditions gave slightly better crystals from 0.2 *M* magnesium acetate, 0.1 *M* Tris, 25%(*v*/*v*) MPD pH 8.2. To try and further improve the crystal morphology, different detergents in the crystallization conditons were evaluated by using the SURF screen from Hampton research using 10 µl sitting drops (5 µl protein in 20 m*M* Hepes, 50 m*M* NaCl pH 8.0 + 4 µl well solution + 1 µl of detergent). The best crystals were obtained by the substitution of 0.05%(*w*/*v*) *β*-octylglucoside in the initial conditions by 1%(*w*/*v*) Mega8. Under these conditions, crystals grew as thin rectangular plates/needles up to 100 × 20 × 1 µm in 8 µl (4 µl protein + 4 µl well solution) hanging drops (Fig. 1). Carefully washed crystals were then analysed by polyacrylamide gel electrophoresis and stained with Coomassie Blue. A single protein band corresponding to a molecular mass of approximately 70 kDa was observed which migrated identically to the starting material, confirming that the whole GST–ERHBD fusion protein had been crystallized (data not shown). Unfortunately, these crystals did not diffract X-rays when frozen at 110 K, using either a Rigaku RU200 rotating anode or synchrotron X-ray sources. (SRS Daresbury, and ESRF Grenoble.) Despite extensive screening of additives and further crystal seeding experiments, there was no further improvement in crystal quality, and no crystals suitable for X-ray structural analysis were obtained.

As part of this study, we used electron microscopy to confirm that the crystals actually contained GST–ERHBD protein. When observed directly in negative stain, the GST–ERHBD crystals appeared rectangular with a clear lattice

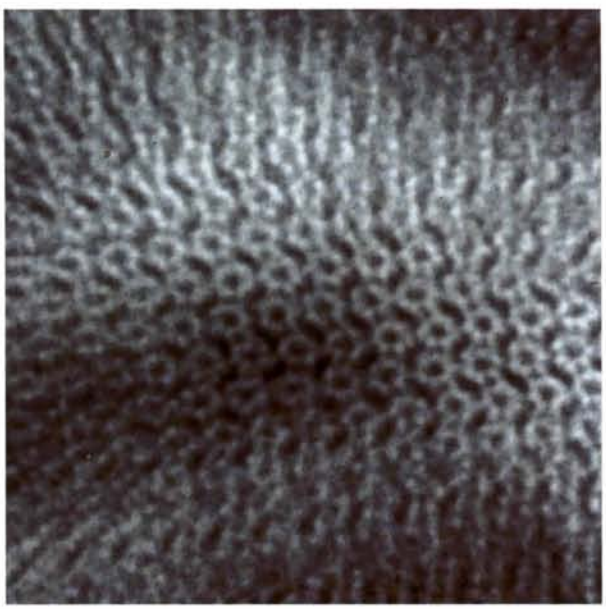

Fig. 2. Electron micrograph of a negatively stained crystal of GST–ERHBD grown from a hanging drop. The lattice of 12.0 × 12.0 nm is visible. Scale bar = 1.0 µm.

(Fig. 2). After noise filtering of the images obtained, we observed trimers of dimeric molecules within the two-dimensional lattice surrounding a central hole (Fig. 3). Unfortunately, the multilayered nature of the crystals precludes the use of electron crystallographic methods to obtain further three-dimensional or improved two-dimensional information. However, the distribution of stain-excluding density indicated that under these conditions the protein monomer has apparent dimensions of approximately 12.0 × 5.0 nm. It has previously been shown by gel-filtration chromatography, that the GST–ERHBD used in this study is dimeric in solution (Arbuckle, 1994). It is possible, therefore, that the noise-filtered EM image of the crystals (Fig. 3) represents the trimeric packing of GST–ERHBD dimers. Since the three-dimensional structures of GST from *S. japonicum* (GST used to make the fusion GST–ERHBD protein; Lim *et al.*, 1994) and the RXRα receptor ligand binding domain (Bourget *et al.*, 1995) are known, we attempted to model a GST–ERHBD fusion protein based on the two known homologous structures (Fig. 4). Our model of the GST–ERHBD fusion also took into account the length of the six-residue peptide linker between the fused proteins, thereby limiting the relative orientations between both molecules. We then manually superposed this model (Cα atoms only) onto the two-dimensional stain-excluding density using the graphics program *PREPI*, avoiding any obvious clashes. The best superposition was obtained when one GST–ERHBD molecule from the dimer was placed in projection on the plane with the second molecule from the dimer lying underneath the plane as shown (Fig. 4). However, given the low resolution of the stain-excluding density map, other orientations of the GST–ERHBD dimer could also be possible. Nevertheless, the model did provide some evidence to suport our EM observations of single GST–ERHBD molecules packed into a crystalline lattice.

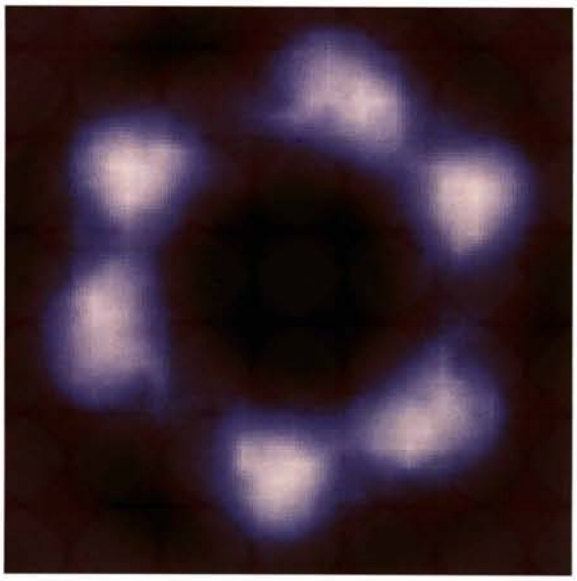

Fig. 3. Unsymmetrized noise-filtered image of a crystalline region of Fig. 2. Protein molecules (white) of approximately 12.0 × 5.0 nm are clearly visible.

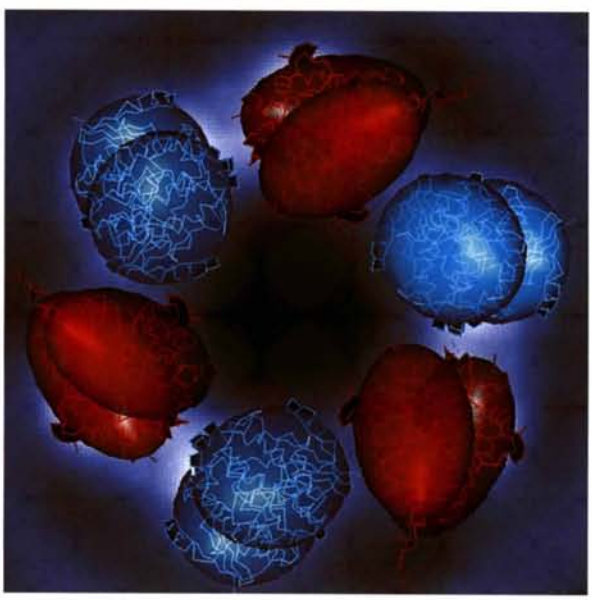

Fig. 4. Model of the *S. japonicum* GST (blue) and ERHBD (red) dimers fitted into the EM stain excluding density. N–C clockwise round the central hole.

There have been a number of suggestions that GST-fusion proteins could be used as a means of obtaining crystals of proteins or peptides which have proven difficult to crystallize alone (Carter *et al.*, 1994). This report shows that it is possible to obtain crystals of large intact fusion proteins. However, in this case, the crystals were not suitable for high resolution X-ray structural analysis, and it remains to be seen whether this approach can lead to high-resolution structures.

Note added in proof: A recent report by Kuge *et al.* (1997) has demonstrated that crystals of the DNA binding domain of DNA-replication-related element binding factor (DREF), obtained as an intact GST–DREF fusion protein, diffract X-rays to 2.5 Å resolution, and a structure determination is in progress. This would seem to confirm that the use of intact fusion proteins for structural studies may be useful in some cases.

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