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Cleaved thioredoxin fusion protein enables the crystallization of poorly soluble ERα in complex with synthetic ligands

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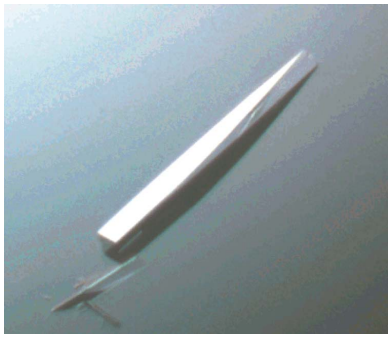

The ligand-binding domain (LBD) of human oestrogen receptor α was produced in *Escherichia coli* as a cleavable thioredoxin (Trx) fusion in order to improve solubility. Crystallization trials with either cleaved and purified LBD or with the purified fusion protein both failed to produce crystals. In another attempt, Trx was not removed from the LBD after endoproteolytic cleavage and its presence promoted nucleation and subsequent crystal growth, which allowed the structure determination of two different LBD–ligand–coactivator peptide complexes at 2.3 Å resolution. This technique is likely to be applicable to other low-solubility proteins.

1. Introduction

Oestrogen receptor (ER) is a major pharmaceutical target for the regulation of fertility and the fight against breast cancer and osteoporosis (McDonnell, 2005). Like the other members of the nuclear receptor superfamily, ER has a multidomain structural organization (Evans, 1988). The physiological hormone and synthetic drugs bind to the ligand-binding domain (LBD) at the carboxy-terminal end of the protein. The LBD has been under intense scrutiny for structure-guided drug design. The difficulty in handling the ER LBD is twofold. Firstly, it contains reactive cysteine residues that can lead to protein oligomerization and aggregation if not taken care of. In particular, Cys530 has been shown to form intermolecular disulfide bonds that produce a tetrameric organization with the carboxy-terminal helix H12 swapped between biologically relevant dimers downstream of these disulfide bonds (Tanenbaum *et al.*, 1998). Reducing agents (Eiler *et al.*, 2001), carboxymethylation (Brzozowski *et al.*, 1997; Kong *et al.*, 2005; Shiau *et al.*, 1998, 2002; Wu *et al.*, 2005) and the mutation of cysteine residues (Gangloff *et al.*, 2001; Renaud *et al.*, 2003, 2005) have successfully been used to prevent protein oxidation. Additionally, the LBD is unstable in the absence of ligand. To address this latter issue, we added the ligand oestradiol-17β during protein synthesis in bacterial culture medium in an attempt to stabilize the LBD (Eiler *et al.*, 2001; Gangloff *et al.*, 2001). Others have purified active protein using oestradiol-affinity chromatography (Brzozowski *et al.*, 1997; Kong *et al.*, 2005; Renaud *et al.*, 2003, 2005; Shiau *et al.*, 1998, 2002; Tanenbaum *et al.*, 1998; Wu *et al.*, 2005). However, in order to crystallize LBD with synthetic ligands of lower affinity than oestradiol-17β and which are only available in small amounts, it was necessary to obtain a source of purified ligand-free LBD. Therefore, we used a soluble fusion to thioredoxin (Trx) in this study.

A small fusion such as a His tag did not hamper the crystallization of human wild-type and unmodified ER LBD (Eiler *et al.*, 2001). With mouse ER LBD (residues 281–599), thin crystals were obtained using the larger GST fusion (Lally *et al.*, 1998). Nevertheless, no subsequent structure has been reported for this carrier-driven GST crystallization technique. Whereas fusion proteins are frequently used for their solubilizing properties and to facilitate purification by affinity chromatography, the presence of a long flexible linker region between the fusion tag and the protein of interest is thought to prevent crystallization because of conformational heterogeneity. Only a limited number of crystal structures of fusion proteins with large affinity tags have been reported to date (for a review, see Smyth *et al.*, 2003).

Table 1
Statistics of ERα LBD–peptide–ligand structure determination.

Values in parentheses are for the highest resolution shell.

Data-collection details (RU68593)†	
Wavelength (Å)	0.979
Unit-cell parameters (Å, °)	a = 56.7, b = 81.8, c = 58.3, β = 111.2
Space group	$P2_1$
Processing statistics	
Resolution range (Å)	30.0–2.25
Unique reflections	22268
Redundancy	3.1 (2.6)
Completeness (%)	94.9 (61.0)
$\langle I/\sigma(I)\rangle$	16.6 (10.0)
$R_{\rm merge}$‡ (%)	6.5 (10.8)

† The data set for RU100132 was isomorphous and of similar quality. ‡ $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$, where $\underline{I_i(hkl)}$ is the ith observed intensity of a measured reflection with Miller indices hkl and $\overline{I(hkl)}$ is the average intensity for this unique reflection.

Here, we present a novel strategy based on the crystallization of the cleaved fusion protein in the presence of the tag moiety. Two crystal structures of ER LBD in complex with a coactivator peptide and two synthetic ligands were solved at 2.3 Å resolution using this technique. Trx appeared to be a critical crystallization agent that promoted nucleation and crystal growth, although it was not present in the crystal itself.

2. Materials and methods

2.1. Construction of the expression vector

The expression plasmid for Trx fused to ERα LBD (Trx-LBD) was constructed from the pET15b-LBD (residue 302–552) expression plasmid described by Eiler *et al.* (2001) with the following modifications: a DNA sequence coding for *Escherichia coli* Trx followed by a His tag and a thrombin-specific cleavage site (LVPRGS; one-letter amino-acid code) was inserted into the *Nde*I site upstream of the LBD sequence. The mutation of Cys530 to alanine (C530A) was performed using the QuikChange Mutagenesis kit according to the manufacturer's instructions (Stratagene). DNA sequencing confirmed the presence of the desired mutation.

2.2. Expression of fusion protein

The Trx-LBD C530A mutant was expressed in BL21(DE3) *E. coli* cells. The culture was carried out in LB medium supplemented with 10%(*w*/*v*) sucrose and 100 µg l^{-1} ampicillin in a 10 l bioreactor (Inceltech) at 310 K until the OD reached 0.5. The culture was then slowly cooled to 293 K before adding 0.5 m*M* IPTG and grown overnight. The cells were harvested by centrifugation and resuspended in 400 m*M* NaCl, 50 m*M* sodium/potassium phosphate pH 7.7.

2.3. Purification

All purification steps were performed at 277 K. The protein purity was analysed by SDS–PAGE and concentrations were measured by UV absorption at 280 nm. Cells were lysed by sonication on ice in 50 m*M* sodium/potassium phosphate, 400 m*M* NaCl, 7 m*M* β-mercaptoethanol pH 7.7 (buffer *A*) and the extract was centrifuged at 14 000*g* for 40 min. The crude extract was loaded onto a 5 ml cobalt-affinity column (Talon Clontech). Nonspecific binding proteins were washed out with 100 ml buffer *A* supplemented with 10 m*M* imidazole pH 7.7. Proteins were eluted with a linear gradient of 20–80 m*M* imidazole over 100 ml. Fractions containing the fusion protein were precipitated overnight by the addition of 2.5 *M* ammonium sulfate. The precipitate was recovered by centrifugation (30 min at 24 000*g*) and dissolved to 10 mg ml^{-1} in 50 m*M* NaCl, 50 m*M* Tris–HCl pH 8.5, 7 m*M* β-mercaptoethanol and 10 µ*M* synthetic ligand. The protein–ligand complex was dialyzed against the same solution for 8 h.

2.4. Cleavage of the fusion protein

Endoproteolytic cleavage of the fusion protein–ligand mixture was achieved using one unit of thrombin (Sigma) per milligram of fusion substrate and incubating at 277 K overnight. The completeness of the proteolytic reaction was assessed by SDS–PAGE. Insoluble aggregates were removed by centrifugation. Following the digestion step, the sample was either used directly in crystallization trials without further purification or subjected to pH precipitation in order to remove the Trx moiety.

2.5. Separation of Trx and LBD by pH precipitation

The soluble digest was dialysed against 50 m*M* NaCl, 50 m*M* PIPES pH 6.0 for 2 h to allow selective precipitation of the LBD. The precipitate was then redissolved to 3–5 mg ml^{-1} in 20 m*M* NaCl, 20 m*M* Tris–HCl pH 8.5, 10 m*M* β-mercaptoethanol.

2.6. Crystallization

For crystallization in the presence and in the absence of Trx, a threefold molar excess of a coactivator peptide (a 15-amino-acid peptide of sequence RHKILHRLLQEGSPS derived from the NR box II of SRC1) was directly added to the sample. The protein was diluted in 50 m*M* NaCl, 50 m*M* Tris–HCl pH 7.5, 10 µ*M* ligand, 7 m*M* β-mercaptoethanol. Crystallization trials were carried out with the hanging-drop technique at 297 K. Hampton Research sparse matrices, as well as specific screens for nuclear receptors (both home-made and from Molecular Dimensions Ltd), were used to determine initial crystallization conditions. Drops were set up by mixing 1 µl protein solution at different concentrations with 1 µl reservoir solution. Small crystals were exclusively obtained in the presence of cleaved Trx. In order to improve their size, seeding was performed. Briefly, microcrystals obtained from an initial crystallization trial were crushed using a microtool spatula and extracted using a nylon cryoloop. The suspension of crushed crystals was then introduced into hanging drops freshly set up against 0.5 ml reservoir solution consisting of 20%(*w*/*v*) PEG 3350, 80 m*M* $MgCl_2$, 100 m*M* Tris–HCl pH 8.5.

2.7. Data collection

Prior to X-ray exposure, crystals were transferred to a cryoprotectant solution consisting of the reservoir solution supplemented with 15%(*v*/*v*) ethylene glycol for 20 s and flashed-cooled in liquid ethane. X-ray data sets were collected at 110 K in a nitrogen-gas stream on European Synchrotron Radiation Facility beamlines ID29 and ID14. The diffraction intensities were processed using *HKL*-2000 and *SCALEPACK* (Otwinowski & Minor, 1997; Table 1).

3. Results and discussion

3.1. Production of Trx-LBD C530A mutant protein

The C530A mutation does not disrupt the transcriptional activity of the nuclear receptor (Reese & Katzenellenbogen, 1991), in contrast to cysteine-to-serine mutations. The latter triggered conformational changes similar to those found in antagonist-bound LBD (Gangloff *et al.*, 2001). We expressed the C530A mutant LBD as a Trx fusion in *E. coli* with a final purification yield per litre of culture of

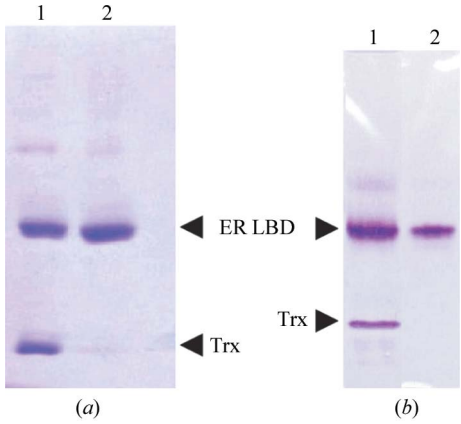

Figure 1
Purified and cleaved Trx-C530A fusion protein used for crystallization trials. (*a*) 12% SDS–PAGE; (*b*) 8–25% native PAGE. In both gels lane 1 contained thrombin-cleaved Trx-C530A and lane 2 contained purified mutant LBD following pH precipitation and Trx elimination.

about 1 mg for the C530A mutant (after removal of the Trx fusion) and 3 mg for the Trx-C530A complex (after cleavage but without removal of the fusion).

3.2. Endoproteolytic cleavage of the fusion protein

Cleavage of the Trx moiety was performed *via* a thrombin site upstream of the LBD sequence and resulted in two peptide chains: the first was Trx His-tagged at its carboxy-terminal extremity and the second was untagged LBD in complex with a ligand. The cleavage was efficient and complete after overnight digestion at 277 K (Figs. 1 and 2). In the absence of the thioredoxin fusion, the apo form tended to aggregate and was not suitable for crystallographic studies. The addition of synthetic oestrogens such as RU68593 and RU100132 (Proskelia) prior to proteolysis was found to stabilize the protein complexes and therefore deemed appropriate for sample preparation.

3.3. Elimination of the Trx moiety

We first attempted to separate the two digestion products using immobilized metal-affinity chromatography on the basis that the His-tagged Trx should interact strongly with the resin, whereas the untagged LBD should flow through. After loading the column, an imidazole gradient was used to wash away protein nonspecifically bound to the column. Contrary to our expectations, the LBD was not detected in the flowthrough and in the early gradient fractions but instead eluted with the His-tagged Trx. This suggests that Trx and LBD interact. Treatment with high salt did not dissociate the complex and Trx and the LBD remained associated when fractionated by ion-exchange chromatography (not shown). A purification technique based on pH precipitation of the LBD enabled the elimination of the soluble Trx in the supernatant (Fig. 1). In contrast to the protein aggregation observed in the absence of ligand, pH precipitation proved to be reversible.

3.4. Crystallization trials in the presence and in the absence of Trx

Crystallization trials using undigested Trx-LBD fusion protein or purified LBD after elimination of the fusion were both unsuccessful. In another attempt, drops were set up using fusion protein processed with thrombin without eliminating the Trx tag. Small needles appeared spontaneously in 20%(*w*/*v*) PEG 3350, 80 m*M* $MgCl_2$, 100 m*M* Tris–HCl pH 8.5 at 297 K. Seeding was required in order to obtain crystals suitable for X-ray analysis (Fig. 2). Protein solution was diluted to different concentrations. The complexes with synthetic oestrogen analogues and coactivator peptide crystallized at low concentrations of 1.2–1.3 mg ml^{-1}. In contrast, Trx is highly soluble. According to Hendrickson and coworkers, Trx from *E. coli* crystallizes at 6.3 mg ml^{-1} and at higher concentrations in the presence of PEG 3350 and cupric acetate at pH 4.2 (Hendrickson *et al.*, 1990). Under the conditions tested, Trx remained in solution and did not interfere with the crystallization of ER LBD bound to a coactivator peptide and to two different agonist ligands. It is surprising that the mixture crystallized in contrast to purified LBD. One possible explanation is that the acid treatment to remove the Trx moiety and to lead to highly pure LBD (Fig. 1) altered the LBD sample, although it appeared to be homogeneous on native PAGE.

3.5. Crystal characterization

Crystals were washed, dissolved and analysed by SDS–PAGE using silver staining (Fig. 2). Whereas the mother liquor contained an equimolar mixture of ERα LBD and Trx, a single band at the molecular weight of the LBD was detected in the crystal. No Trx was present in the crystal. Crystals of LBD in complex with a coactivator peptide and different synthetic ligands are isomorphous and diffracted to 2.3 Å. They belong to space group $P2_1$. The unit-cell parameters (a = 56.7, b = 81.8, c = 58.3 Å, β = 111.2°) are very close to

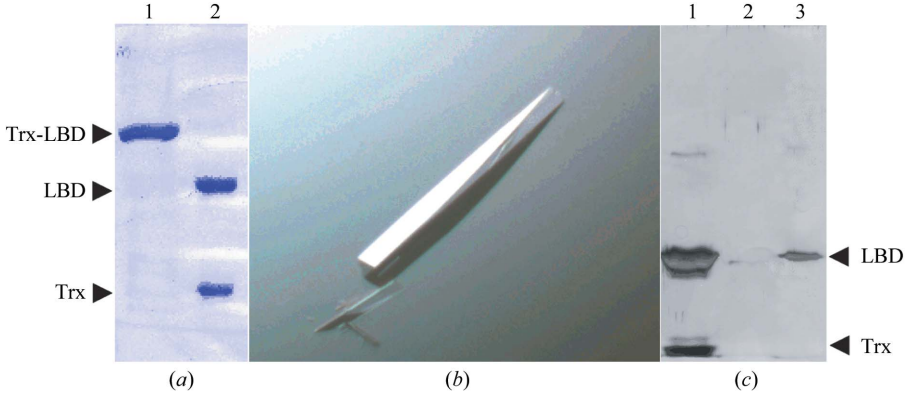

Figure 2
(*a*) 12% SDS–PAGE of purified Trx-C530A LBD before and after cleavage with thrombin. (*b*) Crystal (200 × 40 × 40 µm) of C530A mutant in complex with coactivator peptide and RU68593 obtained in the presence of thioredoxin. (*c*) Silver-stained 12% SDS–PAGE on dissolved crystals. Lane 1, crystallization drop (mother liquor) containing ER LBD (approximately 30 kDa; higher band) and Trx (12 kDa; lower band); lane 2, crystal-transfer liquor; lane 3, dissolved crystal containing only ER LBD.

those of the published structure of human ERα LBD (PDB code 3erd).

The selective crystallization of the LBD suggests that the Trx 'contaminant' was not an issue in obtaining diffraction-quality crystals. Similarly, in chemistry crystallization is routinely used as an efficient purification step for mixtures of small molecules. More importantly, the presence of Trx proved critical for the crystallographic study presented here, as crystals did not appear and/or grow in its absence. This result suggests an important role for Trx as a crystallization adjuvant.

4. Concluding remarks

Fusion partners such as Trx and GST are used to increase the expression levels and the solubility and to facilitate the purification of a protein of interest. In this study, Trx not only helped in the production of an otherwise insoluble protein but also proved critical in the crystallization and therefore the resolution of the atomic structures of two different complexes. To our knowledge, this is the first time Trx has been used as a stabilizing agent in a crystallization experiment. This property is likely to apply to other proteins of limited solubility. This novel technique should be regarded as an interesting alternative for crystallization of difficult proteins.

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