In situ proteolysis of an N-terminal His tag with thrombin improves the diffraction quality of human aldo-keto reductase 1C3 crystals

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Human aldo-keto reductase 1C3 (AKR1C3) stereospecifically reduces steroids and prostaglandins and is involved in the biotransformation of xenobiotics. Its role in various cancers makes it a potential therapeutic target for the development of inhibitors. Recombinant AKR1C3 with a thrombin-cleavable N-terminal His₆ tag was expressed from a pET-28(+) vector for structural studies of enzyme–inhibitor complexes. A modified *in situ* proteolysis approach was applied to specifically remove the His tag by thrombin cleavage during crystallization screening trials. This improved the morphology and diffraction quality of the crystals and allowed the acquisition of high-resolution diffraction data and structure solution. This approach may be generally applicable to other proteins expressed using the pET-28(+) vector.

1. Introduction

Human aldo-keto reductase 1C3 (AKR1C3), also known as 17 β -hydroxysteroid dehydrogenase 5 (17 β HSD5), is an NAD(P)(H)-dependent oxidoreductase that is involved in the stereospecific interconversion of carbonyl groups and alcohols (Adeniji *et al.*, 2013). AKR1C3 reduces steroids and prostaglandins, including the conversions of 4-androstene-3,17-dione to testosterone, of oestrone to 17 β -oestradiol and of prostaglandin D2 to 11 β -prostaglandin F_{2 α} , affecting proliferative signalling pathways that are important in the development of both hormone-dependent and hormone-independent cancers (Byrns *et al.*, 2008; Matsuura *et al.*, 1998; Velić *et al.*, 2009). In addition, AKR1C3 is upregulated in various cancers, including breast and prostate cancer (Adeniji *et al.*, 2013; Qiu *et al.*, 2007), and is involved in the biotransformation of xenobiotics, including drugs and procarcinogens, making this enzyme a potential therapeutic target (Skarydova *et al.*, 2014; Penning, 2015). Large numbers of AKR1C3 inhibitors have been described and some have reached preclinical and clinical phases; however, no AKR1C3 inhibitor has yet been approved. Because the current inhibitors of AKR1C3 often have undesired side effects and may affect off-target metabolic pathways, the development of new inhibitors is required (Penning, 2017).

X-ray crystallographic analysis of complexes of AKR1C3 with candidate inhibitors helps in understanding the interactions between the enzyme and inhibitor on a molecular level

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and in the rational design of new generations of inhibitors. For such studies, a robust procedure for protein production, purification and crystallization is required which yields crystals of very good diffraction quality. To date, there are 43 crystal structures of AKR1C3 in complex with inhibitors or natural substrates in the Protein Data Bank (Berman *et al.*, 2000). The first AKR1C3 structures were solved using three different protein constructs: an untagged construct (Komoto *et al.*, 2004, 2006), a construct with a noncleavable C-terminal histidine tag (Lovering *et al.*, 2004) and a glutathione *S*-transferase (GST) fusion construct (Qiu *et al.*, 2004, 2007), where the GST was removed before crystallization. However, by far the vast majority of AKR1C3 structures reported used a noncleavable histidine tag (His₆) at the C-terminus of the protein (Flanagan *et al.*, 2012, 2014; Jackson *et al.*, 2012; Jamieson *et al.*, 2012; Heinrich *et al.*, 2013; Amano *et al.*, 2015). Several AKR1C3 structures with an uncleaved N-terminal His₆ tag have also been determined recently (Zhao *et al.*, 2015).

In situ proteolysis is a method that is used for the rescue or improvement of protein crystallization and the diffraction quality of crystals. In this method, small amounts of broad-specificity proteases are added to the protein solution immediately before crystallization. The proteolytic removal of flexible parts of the protein molecule is believed to facilitate the formation of crystal contacts (Dong *et al.*, 2007; Wernimont & Edwards, 2009).

Here, we report a simple and economic *in situ* proteolysis approach using thrombin for the specific removal of the N-terminal His-tag sequence from a pET-28(+) AKR1C3 protein construct during crystallization. This approach yielded crystals with improved diffraction quality compared with those grown using His-tagged protein.

2. Materials and methods

2.1. Protein expression and purification

Plasmid DNA encoding human AKR1C3 in a pET-28b(+) bacterial expression vector was generously provided by Professor Chris Bunce (University of Birmingham). The resulting recombinant protein contains an N-terminal His₆ tag followed by a recognition site for thrombin protease (underlined): MGSSHHHHHHSSGLVPRGSH. The expression and purification of recombinant AKR1C3 were conducted as described previously (Lovering *et al.*, 2004; Velić *et al.*, 2009). Briefly, *Escherichia coli* BL21(DE3) cells transformed with the AKR1C3-pET-28 plasmid were cultivated at 310 K in LB medium with 50 µg ml⁻¹ kanamycin to an OD_{595 nm} of ~0.6, induced with isopropyl β-D-1-thiogalactopyranoside at a final concentration of 0.5 mM and further cultivated at 293 K for approximately 18 h. The cells were harvested and resuspended in buffer A (25 mM sodium phosphate pH 8.0, 150 mM NaCl), lysed by freeze-thaw/sonication in the presence of lysozyme (1 mg ml⁻¹; Sigma–Aldrich, USA) and clarified by centrifugation (30 min, 10 000g). The clarified lysate was applied onto a 1 ml HisTrap nickel column (GE Healthcare, USA) equilibrated in buffer A and the recombinant AKR1C3 was eluted

with 500 mM imidazole in buffer A. The pooled AKR1C3-containing fractions were then applied onto a P10 desalting column (Bio-Rad, USA) for buffer exchange into buffer B (10 mM potassium phosphate pH 7.0, 1 mM EDTA, 1 mM DTT). Purified AKR1C3 was concentrated, supplemented with 1.2 mM NADP⁺ (Sigma–Aldrich, USA) and stored in aliquots at 203 K; the final protein concentration was 24 mg ml⁻¹. For crystallization trials, a potential steroidal AKR1C3 inhibitor (unpublished work) was also added to a concentration of 0.4 mM from a 10 mM stock in 100% DMSO. This protein sample was then thawed and additionally purified on a HiLoad 16/600 Superdex 200 pg column equilibrated in buffer B using an ÄKTA Basic FPLC system (GE Healthcare, USA). Purified AKR1C3 was concentrated and supplemented with 1.2 mM NADP⁺ (Sigma–Aldrich, USA) and 0.4 mM AKR1C3 inhibitor before storage in aliquots at a concentration of 31 mg ml⁻¹ at 203 K. Protein purity was monitored by SDS–PAGE with Coomassie Brilliant Blue staining and the protein concentration was quantified using the Bradford colorimetric assay.

2.2. Protein crystallization

AKR1C3 at a concentration of 31 mg ml⁻¹ in 10 mM potassium phosphate pH 7.0, 1 mM EDTA, 1 mM DTT supplemented with 1.2 mM NADP⁺ (Sigma–Aldrich, USA) and 0.4 mM AKR1C3 inhibitor (unpublished work) was used for initial crystallization screening. Screening was performed at 291 K by sitting-drop vapour diffusion using Gryphon (Art Robbins Instruments, USA) and Oryx8 (Douglas Instruments, UK) crystallization workstations in 96-well sitting-drop MRC 3-well plates (Jena Bioscience, Germany). The commercial screening kits Morpheus (Molecular Dimensions, UK) and The PEGs Suite (Qiagen, USA) were tested. A total volume of 300 nl of a mixture of protein and precipitant solutions in 1:1, 2:1 and 1:2 volume ratios was equilibrated against 30 µl reservoir solution. The crystallization process was monitored using a Minstrel DT UV automated imaging system with a Gallery DT plate hotel (Rigaku, Japan) and an Olympus SZX10 optical microscope (Olympus, Japan).

2.3. *In situ* proteolysis

Bovine thrombin (catalogue No. T4648-1KU; Sigma–Aldrich, USA) was dissolved in distilled water to a concentration of 2.7 mg ml⁻¹ and 8 µl of this solution was added to the AKR1C3 solution (200 µl) immediately before crystallization. The final concentration of thrombin in the protein sample used for crystallization was 0.1 mg ml⁻¹ and the molar ratio of AKR1C3 to thrombin was 280:1.

2.4. N-terminal protein sequencing

N-terminal amino-acid sequences were determined by Edman degradation using a Procise Protein Sequencing System (494 cLC Protein Sequencer, Applied Biosystems, USA). In each sequencing cycle, the N-terminus of AKR1C3 was treated with phenylisothiocyanate and the resulting

stabilized phenylthiohydantoin amino-acid derivative was analyzed by reversed-phase HPLC.

2.5. Diffraction data collection and processing

The crystals grown using the Morpheus kit were harvested without additional cryoprotection. The crystals grown using The PEGs Suite were cryoprotected by the addition of glycerol directly to drops with crystals to a final concentration of 20%(v/v) immediately before harvesting. Diffraction quality was tested at 100 K on an in-house MicroMax-007 HF Microfocus X-ray generator with a VariMax VHF Arc)Sec confocal optical system (Rigaku, Japan), an AFC11 partial- χ four-axis goniometer (Rigaku, Japan), a PILATUS 300K detector (Dectris, Switzerland) and a Cryostream 800 cryo-cooling system (Oxford Cryosystems, England). A full diffraction data set was collected at 100 K on MX 14.1 operated by the Joint Berlin MX-Laboratory at the BESSY II electron-storage ring in Berlin-Adlershof, Germany (Gerlach *et al.*, 2016). Diffraction data were processed using XDS (Kabsch, 2010).

2.6. Structure solution

The structure of human AKR1C3 was solved by molecular replacement with *MOLREP* (Vagin & Teplyakov, 2010) using the structure of human AKR1C3 (PDB entry 1zq5; Qiu *et al.*, 2007) as the search model. Initial model refinement was performed using *REFMAC5* (Murshudov *et al.*, 2011) from the CCP4 suite (Winn *et al.*, 1994).

3. Results and discussion

We expressed AKR1C3 from a pET-28b(+) vector, resulting in recombinant protein containing an N-terminal His₆ tag followed by a thrombin-recognition sequence. The AKR1C3 protein (molecular weight 39 kDa) was purified by a combination of nickel-affinity and size-exclusion chromatography, with a yield of 20 mg per litre of *E. coli* cell culture. Based on size-exclusion chromatography, the apparent molecular weight of recombinant AKR1C3 corresponded to a monomer (data not shown), which is the 'biological unit' of this enzyme (Penning *et al.*, 2000). The protein purity was >95% as assessed by Coomassie Brilliant Blue staining following SDS-PAGE (data not shown).

In previous reports, AKR1C3 constructs with an N-terminal His tag were routinely used for enzymatic assays, while structural analyses were performed using constructs with a C-terminal His tag (Jamieson *et al.*, 2012). Only three of the 43 AKR1C3 structures in the PDB were determined using a construct with an N-terminal His tag, with resolutions of 2.0 and 2.3 Å (Zhao *et al.*, 2015), while 29 of the AKR1C3 structures were solved using a C-terminally His-tagged construct, with resolutions ranging from 1.2 Å, which was the best resolution achieved for AKR1C3 (Lovering *et al.*, 2004), to 2.8 Å, with an average resolution of 1.9 Å. Three AKR1C3 structures were solved from a GST-fusion construct, where the GST was removed before crystallization (Qiu *et al.*, 2004,

2007), and the remaining structures originated from untagged protein. Moreover, in most space groups the N-terminus is located in close proximity to a neighbouring molecule in the crystal lattice, although the first four or five N-terminal residues are usually missing from the models. Based on these facts, we suspected that the presence of an N-terminal His tag could affect crystal formation and/or diffraction.

Therefore, in the present study we modified an *in situ* proteolysis approach generally used for the nonspecific cleavage of unstructured extensions or flexible regions of proteins in order to promote crystallization (Wernimont & Edwards, 2009; Dong *et al.*, 2007). We used thrombin protease to target its recognition sequence in our protein construct and specifically remove the N-terminal His tag *in situ* during crystallization trials. This is a time- and cost-effective approach relative to the standard tag-cleavage procedure, in which a relatively high amount of His-tagged thrombin is used and an additional purification step is required.

3.1. *In situ* His-tag proteolysis improves crystal morphology

Two sets of crystallization trials using The PEGs Suite (Qiagen, USA) and Morpheus (Molecular Dimensions, UK) were conducted with AKR1C3 in the presence and absence of a trace amount of thrombin; the molar ratio of AKR1C3 to thrombin was 280:1. Crystallization hits were observed in numerous conditions in both crystal screens and the majority of crystals appeared overnight (Fig. 1). There was no significant difference in the number of conditions that yielded crystals between samples with or without thrombin (17 and 16, respectively; Fig. 1). However, the crystals grown with and without thrombin differed in morphology, as assessed by visual comparison of the crystals. Some conditions yielded needle-shaped microcrystal clusters, while other conditions yielded large harvestable crystals. The crystals were usually three-dimensional and elongated, and were often inter-grown with multiple nucleation layers (Fig. 1). Crystals grown from the sample treated with thrombin contained visibly fewer nucleation layers. These crystals were also easier to separate from clusters during harvesting, or it was at least possible to centre their monocrystalline parts in the X-ray beam for diffraction analysis.

3.2. *In situ* His-tag proteolysis improves diffraction quality

All harvestable crystals were mounted in loops, cryoprotected if grown using The PEGs Suite and flash-cooled in liquid nitrogen. All successfully harvested crystals were subjected to X-ray diffraction tests on an in-house diffractometer (Fig. 1). Crystals grown in the presence of thrombin provided diffraction images with generally better properties as assessed by visual comparison. In particular, the diffraction data showed a higher resolution, a better spot shape and a higher prevalence of spots from a single crystal lattice, which facilitated diffraction data indexing.

Crystals obtained from The PEGs Suite required cryoprotection, and thus their diffraction quality may be affected by this further manipulation. The crystals grown using the

Morpheus kit did not require additional cryoprotection (Gorrec, 2009), and some of the Morpheus conditions yielded crystals from both samples with and without added thrombin. Therefore, we could make a direct pairwise comparison of crystal morphology and diffraction quality in several cases (Fig. 2). This comparison clearly demonstrates that *in situ* His-tag removal using a limited amount of thrombin resulted in improved crystal morphology and diffraction quality in terms of resolution limit, spot size and spot shape.

3.3. *In situ* thrombin proteolysis is efficient in precipitant solutions

Crystallization conditions typically contain mixtures of various precipitants, including high salt concentrations, high concentrations of high-molecular-weight polyalcohols *etc.* Since such solutions do not provide optimal cleavage conditions for thrombin, we analyzed crystals grown in the Morpheus screen from samples treated *in situ* with thrombin

by Edman N-terminal sequencing. The sequencing results, together with the compositions of the corresponding crystallization solutions, are listed in Table 1. Control sequencing was performed on the protein sample that had not been treated with thrombin and the N-terminal sequence GSSHHH-HHHSSG was identified, which corresponds to the N-terminal His₆ tag. The His tag is followed by the sequence LVPRGSH, which is expected to be cleaved by thrombin between R and G. Analysis of the crystallization results clearly indicates that thrombin efficiently removed this His tag *in situ* in the crystallization cocktails. We expect that this approach will be generally applicable for constructs derived from the pET-28(+) vector.

3.4. Structure determination

Crystals obtained from well D1 of the Morpheus screen [10%(w/v) PEG 20 000, 20%(v/v) PEG MME 550, 0.02 M of each alcohol [1,6-hexanediol, 1-butanol, (*RS*)-1,2-propanediol,

Figure 1

Crystallization screening results from Morpheus (top) and The PEGs Suite (bottom). A schematic representation of the crystallization plates is shown; the colours indicate wells which yielded crystals in the absence (yellow) and the presence (green) of thrombin. The values in the specific wells indicate the best achieved resolution (in Å) in diffraction tests on crystals that could be harvested. Representative images of crystals are shown below the schemes, labelled by well number, protein:precipitant volume ratio (1:2, 1:1 or 2:1) and the absence (-T) or presence (+T) of thrombin.

2-propanol, 1,4-butanediol and 1,3-propanediol], 0.1 M MES-imidazole pH 6.5] diffracted to 2.0 Å resolution on the in-house X-ray source and were subsequently analyzed using synchrotron radiation, yielding a diffraction data set at a resolution of 1.5 Å. Diffraction data were collected at 100 K on MX 14.1 operated by the Joint Berlin MX-Laboratory at the BESSY II electron-storage ring in Berlin-Adlershof, Germany (Gerlach *et al.*, 2016). Data-collection statistics are given in Table 2.

Data were collected to a high-resolution limit of 1.5 Å. Only four structures of the 43 AKR1C3 structures in the PDB have a resolution of or below 1.5 Å; these include two structures determined using a construct with a C-terminal His tag (Lovering *et al.*, 2004) and two structures determined using a GST-fusion construct, where the GST was removed before

Table 1

Edman sequencing of AKR1C3 crystals grown in the presence of thrombin.

Well No.	Crystallization solution	N-terminal sequence†
D1	10% (w/v) PEG 20 000, 20% (v/v) PEG MME 550, 0.02 M of each alcohol [1,6-hexanediol, 1-butanol, (<i>RS</i>)-1,2-propanediol, 2-propanol, 1,4-butanediol and 1,3-propanediol], 0.1 M MES-imidazole pH 6.5	GSHMDSXQQXV
D4	12.5% (w/v) PEG 1000, 12.5% (w/v) PEG 3350, 12.5% (v/v) MPD, 0.02 M of each alcohol [1,6-hexanediol, 1-butanol, (<i>RS</i>)-1,2-propanediol, 2-propanol, 1,4-butanediol and 1,3-propanediol], 0.1 M MES-imidazole pH 6.5	GSHXDSXQ
F1	10% (w/v) PEG 20 000, 20% (v/v) PEG MME 550, 0.02 M of each monosaccharide (D-glucose, D-mannose, D-galactose, L-fucose, D-xylose and N-acetyl-D-glucosamine), 0.1 M MES-imidazole pH 6.5	GSHMDSXQQXV

† Residues labelled X could not be identified, the methionine corresponds to the first amino acid of AKR1C3 and the preceding GSH are residues that remain following thrombin cleavage.

crystallization (Amano *et al.*, 2015). Of the comparatively few AKR1C3 structures determined using an N-terminally His-tagged construct, the best has a reported resolution of 2.0 Å (Zhao *et al.*, 2015). The majority of AKR1C3 structures in the PDB, including those with resolutions of or below 1.5 Å, belong to space group $P2_12_12_1$. Our crystal exhibited $P1$ space-group symmetry, the second most frequent AKR1C3 space group, represented by nine AKR1C3 structures in the PDB. All structures resulting from N-terminally His-tagged AKR1C3 constructs belong to space group $P1$ (Zhao *et al.*, 2015), but better resolved $P1$ structures were generally determined using untagged protein, with a best resolution of 1.69 Å (Komoto *et al.*, 2004; Chen *et al.*, 2012; Liedtke *et al.*, 2013).

Figure 2

Morphology and diffraction-quality comparison of crystals produced in the absence (left) and presence (right) of thrombin in Morpheus screen conditions (a) D1 with a protein:precipitant drop ratio of 1:1, (b) D4 with a protein:precipitant drop ratio of 1:1 and (c) F1 with a protein:precipitant drop ratio of 1:2.

Table 2

Diffraction data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.9184
Space group	<i>P</i> 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	39.36, 51.43, 75.96
α , β , γ (°)	76.88, 84.26, 77.69
Resolution range (Å)	45.59–1.50 (1.54–1.50)
No. of unique reflections	87396 (5588)
Completeness (%)	96.3 (83.4)
Multiplicity	3.3 (2.8)
Average <i>I</i> / σ (<i>I</i>)	4.07 (0.76)
<i>R</i> _{meas} [†] (%)	17.0 (89.1)
CC _{1/2} [‡]	98.6 (53.6)
Overall <i>B</i> factor from Wilson plot (Å ²)	25.76

[†] $R_{\text{meas}} = \sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the observed and average intensities, respectively, and $N(hkl)$ is the multiplicity of reflection hkl . [‡] Pearson's correlation coefficient determined using the data set randomly split in half.

The triclinic crystal contained 37% solvent and two protein molecules in the asymmetric unit. The structure of human AKR1C3 in complex with NADP⁺ and a potential AKR1C3 inhibitor (unpublished data) was determined at a resolution of 1.5 Å by molecular replacement using phases derived from a previously solved human AKR1C3 structure available in the PDB as entry 1zq5 (Qiu *et al.*, 2007). After an initial round of refinement, the *R* factors decreased to *R* = 26.5% and *R*_{free} = 29.4% and the quality of the initial electron-density map (Fig. 3) indicate correct structure solution.

We did not proceed further with refinement of the model because no electron density indicating the presence of inhibitor in the active site of the enzyme was observed. Further data sets will be collected from other crystals obtained from this crystallization screening, and co-crystallization optimization will also be performed in order to find conditions that are optimal for the structure determination of protein–inhibitor complexes.

We have modified the *in situ* proteolysis technique to specifically remove the N-terminal His tag from recombinant

Figure 3

Initial electron-density map for AKR1C3. The $2F_o - F_c$ electron-density map after the first round of refinement is shown contoured at 1σ .

AKR1C3 protein using thrombin during a crystallization screening trial. This approach resulted in crystals with improved morphology and diffraction quality. We have determined the three-dimensional structure of AKR1C3 in space group *P*1 at 1.5 Å resolution, which is the best resolution so far achieved for AKR1C3 in this crystal form. *In situ* proteolytic His-tag removal by thrombin is a simple and economic procedure that may be generally applicable to the crystallization of proteins expressed from pET-28(+)-derived vectors.

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