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Production of crystals of human aldose reductase with very high resolution diffraction

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As the action of human aldose reductase (hAR) is thought to be linked to the pathogenesis of diabetic complications, much effort has been directed towards the analysis of the catalytic mechanism and the development of specific inhibitors. Here, the crystallization of recombinant hAR with its cofactor $NADP^+$ at 277 K in the presence of the precipitating agent PEG 6000 is reported. The crystals diffract to high resolution (1.1 Å) and belong to the $P2_1$ space group with unit-cell parameters $a = 49.97$, $b = 67.14$, $c = 48.02$ Å, $\beta = 92.2^\circ$ with one molecule per asymmetric unit. Seleno-substituted hAR crystals were also produced and diffract to 1.7 Å on a conventional X-ray source.

1. Introduction

Aldose reductase (alditol-NADPH-1-oxidoreductase; E.C. 1.1.1.21), a member of the aldo-ketoreductase superfamily, catalyses the NADPH-dependent reduction of a wide range of aldehyde substrates to their corresponding alcohols. The crystallographic structures of the porcine and human aldose reductase are similar and consist of a single domain folded into an eight-stranded α/β barrel (Rondeau *et al.*, 1992; Wilson *et al.*, 1992). Although the normal physiological role of aldose reductase has not yet been established, this enzyme is part of the polyol pathway and its action seems to be linked to the development of long-term complications of diabetes mellitus such as cataracts, retinopathy and neuropathy (Larson *et al.*, 1988). Therefore, the design of inhibitors is of great interest and structural information about the active site is helpful in the search for more efficient molecules. We have previously conducted an investigation using porcine aldose reductase from a non-recombinant source. In the context of inhibitor design (Urzhumtsev *et al.*, 1997), a structural study of the human enzyme was undertaken to provide information about the active site at the atomic level.

2. Materials and methods

2.1. Overproduction and purification of recombinant hAR

The open reading frame of the *hAR* gene (Accession GenBank/EMBL Data Bank Number J05017) was amplified by PCR from cDNA (Chung & La Mendola, 1989) and cloned into the T7 RNA polymerase-based vector pET15b (Novagen). Expression of the $(his)_6$–hAR in the *E. coli* strain BL21(DE3) (Novagen) is induced by IPTG (Euromedex) during a 3 h culture at 310 K. The pellet from a 4 l culture was disrupted by sonication and centrifuged. The supernatant was applied on a Talon metal-affinity column (Clontech). After thrombin cleavage of the hexahistidine extension, the detagged protein was then loaded on a DEAE Sephadex A-50 column (Pharmacia) and eluted with an NaCl gradient.

2.2. Mass spectrometry

Mass spectrometric studies were performed on an electrospray (ES) ionization triple quadrupole mass spectrometer (VG BioQ, Micromass), upgraded by the manufacturer so that the source and the first quadrupole (Q1) have Quattro II performances [mass-to-charge ratio (m/z) range of 4000] and the second quadrupole (Q2) has an extended m/z range (up to 8000). For spectra acquired above m/z 4000, Q1 operates as a high-pass filter in the radio-frequency-only mode to transmit high mass ions to the second quadrupole.

The accurate molecular weight of hAR was deduced from the wide distribution of highly charged states observed in the ES mass spectrum of the denatured enzyme [5 µ*M* in a 1:1(*v*:*v*) water–acetonitrile mixture containing 1% formic acid]. For the observation of the oligomeric forms, hAR was prepared in near-physiological conditions (10 µ*M* in 10 m*M* ammonium acetate pH 7.0) and was then desalted prior to mass spectrometric measurements by a five dilution–concentration step dialysis using Centricon ten concentrators.

Table 1
Intensity statistics as a function of resolution for the diffraction data collected at 103 K from a crystal of native aldose reductase.

D_{min} (Å)	D_{max} (Å)	R_{sym}	Completeness (%)	$\langle I \rangle$	$\langle \sigma(I) \rangle$	Multiplicity
15.00	2.37	0.017	95.4	44119.6	946.4	2.93
2.37	1.88	0.027	96.3	15176.6	416.8	2.97
1.88	1.64	0.044	95.3	6169.0	257.0	2.97
1.64	1.49	0.068	94.4	3731.1	279.0	2.97
1.49	1.39	0.109	93.5	2443.1	250.6	2.95
1.39	1.30	0.144	92.9	1896.8	249.9	2.94
1.30	1.24	0.187	91.9	1556.5	255.0	2.93
1.24	1.18	0.218	91.1	1353.3	263.0	2.94
1.18	1.14	0.279	89.8	1125.4	264.1	2.88
1.14	1.10	0.357	89.3	823.6	259.1	2.89
All		0.043	93.0	8085.5	347.5	2.94

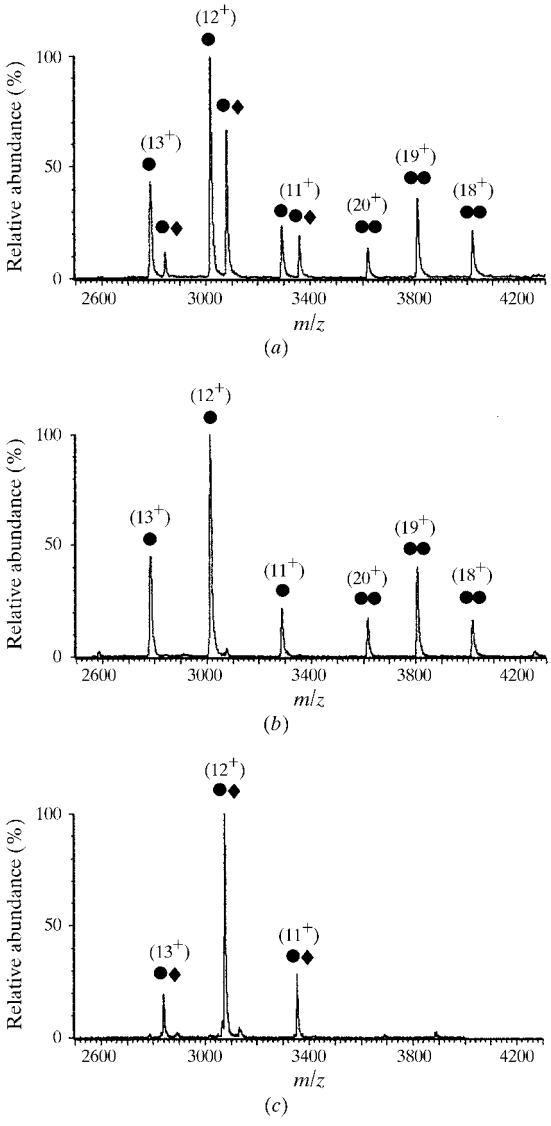

Figure 1
ES mass spectra of hAR recorded under non-denaturing conditions, showing (*a*) a mixture of hAR apoenzyme [present in both monomeric (●) and dimeric (●●) forms] and hAR holoenzyme (●♦), as obtained after the purification steps described in §2, (*b*) the homogeneous apoenzyme (again present in both monomeric and dimeric states), obtained from the mixture shown in (*a*) after supplementary washes on a Talon metal-affinity column, (*c*) the pure holoenzyme, reconstructed from the apoenzyme in (*b*) by adding the coenzyme NADP$^+$.

The mass spectra presented in this paper are an averaged sum of ten scans from m/z 600 to 2000 at a scan rate of 6 s scan^{-1} for mass measurement under denaturing conditions, and from m/z 2400 to 5500 at a scan rate of 10 s scan^{-1} for the observation of the native enzyme. The instrument was calibrated over the m/z range 600–2000 by using the multiply charged ions produced by horse heart myoglobin (Sigma) dissolved in aqueous acetonitrile, and over the m/z range 2400–5500 by using the multiply charged ions produced by hen egg-white lysozyme (Sigma) dissolved in water.

2.3. Crystallization trials and preliminary crystallographic analysis

All trials were carried out in Linbro 24-well tissue-culture plates (Flow Laboratories) using the hanging-drop vapour-diffusion method. The enzyme was dialyzed against 50 m*M* ammonium citrate pH 5.0. The drops, of final volume 12 µl, containing 15 mg ml^{-1} hAR with two equivalents of the oxidized form β-NADP$^+$ (Sigma) and 5% PEG 6000, were equilibrated against a well containing 120 m*M* ammonium citrate pH 5.0, 20% PEG 6000. Crystals were grown at 277 K.

3. Results and discussion

3.1. Overproduction and purification

Recombinant hAR was overproduced in *E. coli* in a soluble form with an hexahistidine N-terminal extension $(his)_6$–hAR. Using the purification scheme described in §2, we obtained an highly purified enzyme with a molecular weight of 36138 ± 1 Da measured by mass spectrometry under denaturing conditions. This value corresponds to the weight derived from the peptidic sequence (M_r = 36134.8 Da) of the enzyme starting as expected with GSHM, the four residues remaining from the hexahistidine N-terminal extension after thrombin cleavage.

The Michaelis constants for the purified enzyme with D-xylose as a substrate showed that the enzyme is fully active (k_{cat} = 0.5 s^{-1} and K_m = 6 µ*M*); these data are similar to those determined with the same procedure reported in the literature (Bohren *et al.*, 1992).

3.2. Preparation of the holoenzyme

hAR forms a stable non-covalent complex with the NADPH cofactor. To detect the presence of the coenzyme in our preparations, the protein was analyzed by mass spectrometry under non-denaturing conditions. A typical ES mass spectrum acquired for hAR prepared under near-physiological conditions is shown in Fig. 1(*a*). The enzyme appears to be present in solution as a mixture of apoenzyme (12-fold charged state observed at m/z 3012) and holoenzyme (12-fold charged state observed at m/z 3074). Fig. 1(*a*) also shows that, as described for the porcine enzyme, the hAR apoenzyme exists in solution as an equilibrium between a monomeric form (12-fold charged state observed at m/z 3012) and a dimeric form (19-fold charged state observed at m/z 3805; Potier *et al.*, 1997). It should be emphasized that the relative amount of apo and holoenzyme varies from batch to batch. In particular, the proportion of apoenzyme increases when the protein bound to the affinity column is washed with an increasing volume of buffer (Fig. 1*b*). The apoenzyme can be converted into the holoform by adding one equivalent of NADP$^+$ (Fig. 1*c*).

The mass spectrometry results show clearly that while the apoenzyme form appears as a stable dimer, the holoenzyme form does not (Figs. 1*b* and 1*c*). This is true for both the pig enzyme and the human enzyme, as shown in previous studies (Potier *et al.*, 1997).

3.3. Crystallization of hAR

As the mass-spectrometry measurements are performed in a vacuum, the electrostatic interactions are strengthened compared with the hydrophobic interactions and therefore potential aggregates are difficult to visualize. To complete our study, gel-filtration chromatography and dynamic light-scattering experiments were used to check that the solution was monomeric and suitable for crystallization experiments (data not shown).

The holo-hAR was crystallized at 277 K in the presence of the precipitating agent PEG

Figure 2
Crystal of hAR. Growth conditions are as given in §2.

6000 in 50 m*M* ammonium citrate pH 5.0, as described in §2. Typical crystals grew in the presence of protein precipitate and reached dimensions 600 × 400 × 100 µm within a week. As shown in Fig. 2, they are composed of thin plates stacked together which could be separated without affecting the diffraction pattern. The crystals were stabilized with 25% PEG 6000 in 120 m*M* ammonium citrate pH 5.0 and diffract to a resolution limit of 1.1 Å at 103 K using a synchrotron X-ray source (see Table 1). They belong to space group $P2_1$ with unit-cell parameters $a = 49.97$, $b = 67.14$, $c = 48.02$ Å, $\beta = 92.2^\circ$.

In order to develop a rational analysis for the design of specific inhibitors of hAR, it is important to understand the mechanism of the enzymatic catalysis, which proceeds through a proton-transfer step. The availability of crystals which diffract to 1.1 Å resolution will give high-quality experimental data. The use of seleno-substituted crystals in conjunction with MAD technology should allow us to visualize the atomic orbitals and to analyze the protonation state of the residues involved in catalysis. As a first step, seleno-substituted hAR was produced using protocols described in the literature (Budisa *et al.*, 1995) and the same purification and crystallization protocol was used as for native hAR. Crystals are isomorphous to the native and preliminary tests using the laboratory source show the same quality of diffraction.

4. Conclusions

We have reported the crystallization of native and seleno-substituted holo hAR. The main difference observed thus far between the previously studied pig AR and the human AR studied here is the very high resolution of the diffraction pattern. This result, together with diffraction data from seleno-substituted hAR collected at various wavelengths, should allow a detailed analysis of the proton-transfer mechanism and of the inhibitor-binding site.

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