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Well Ordered Crystals of a Short-Chain Alcohol Dehydrogenase from *Drosophila lebanonensis*: Re-evaluation of the Crystallographic Data and Rotation-Function Analysis

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Abstract

Alcohol dehydrogenase prepared from *Drosophila lebanonesis* yields well ordered plate-like crystals which diffract to better than 2.3 Å resolution. The crystals belong to space group $P2_1$ of the monoclinic system; the unit-cell dimensions are $a = 65.25$, $b = 55.77$, $c = 70.02$ Å, $\alpha = 90$, $\beta = 107.08$, $\gamma = 90°$. The asymmetric unit of the crystal cell is most probably occupied by a dimer, corresponding to a packing density of 2.15 Å^3 Da^{-1}. The orientation of the non-crystallographic twofold symmetry axes is determined by analysis of a self-rotation function calculated with native intensity data.

Introduction

Drosophila alcohol dehydrogenase (ADH, E.C. 1.1.1.1) catalyzes the conversion of primary and secondary alcohols to aldehydes and ketones. The so-called 'short chain' enzymes are not structurally related to the 'medium chain' zinc dehydrogenases which catalyze the same reaction in all other eukaryotes analyzed so far, including yeast, higher plants and vertebrates (Jörnvall, Persson & Jeffrey, 1981).

ADH has been widely characterized at the biochemical and genetic level in many different species of the genera. So much information has been gathered on the ADH gene–enzyme system that it now constitutes a paradigm to address fundamental questions concerning eukaryotic gene expression, molecular evolution and the adaptive value of natural polymorphisms (van Delden, 1982; Sullivan, Atkinson & Starmer, 1990).

The active form of the enzyme is a dimer of identical subunits ($M_r = 27\,000$). It requires NAD$^+$ as a cofactor, prefers short aliphatic alcohols as substrates and is zinc independent. Due to structural homologies with other enzymes of the short-chain dehydrogenase family, which comprises among others, mammalian prostaglandin dehydrogenases and carbonyl reductases (Persson, Krook & Jörnvall, 1991), *Drosophila* ADH has been incorporated as a member of this family.

Whereas liver ADH has been crystallized some time ago (Eklund *et al.*, 1976) and its structure has helped to postulate the catalytic mechanism of the medium-chain enzymes, the only short-chain dehydrogenase characterized so far, 3α-20β-hydroxysteroid dehydrogenase (Ghosh *et al.*, 1991), cannot provide a reasonable model to approach the catalytic function of *Drosophila* ADH. The main reasons for this are a relatively low level of homology between them (about 25% identical residues), the number of polypeptide chains involved in the native forms, tetramer *versus* dimer, and the substrate specificity. 3α-20β-hydroxysteroid dehydrogenase is involved in the oxidation of the hydroxyl group of androstane and pregnane derivatives, whereas *Drosophila* ADH catalyzes the oxidation of short aliphatic chains of primary and secondary alcohols.

The present study has been carried out with ADH from *Drosophila lebanonensis*, a species of the Scaptodrosophila radiation. The enzyme from this species has been characterized at the biochemical (Winberg, Hovik, McKinley-McKee, Juan & Gonzàlez-Duarte, 1986), structural (Vilarroya, Juan, Egestad & Jörnvall, 1989), and gentic level (Albalat & Gonzàlez-Duarte, 1993) and has been compared with many other *Drosophila* species.

The crystallization of *Drosophila* ADH under similar conditions has been described (Gordon, Bury, Sawyer, Atrian & Gonzàlez-Duarte, 1992). The crystals have been assigned to space group $P2_1$, the crystal parameters, however, may be doubted and are not in agreement with the analysis presented in this paper. More specifically, Gordon *et al.* (1992) have published a unit cell with cell parameters of $a =$

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81.24, $b = 55.75$, $c = 109.60$ Å, $\beta = 94.26°$, which is in fact a unit cell corresponding to a superlattice with face centering and is almost twice as large in volume compared to the cell which is described in this paper. These authors have furthermore concluded that the asymmetric unit of the monoclinic cell is occupied by an ADH tetramer and that their crystals were often twinned and not useful for diffraction analysis.

We have improved the crystallization conditions by a broad screening and carefully reinvestigated the crystallographic parameters with the aim to define the smallest possible unit cell. The knowledge of the three-dimensional structure of *Drosophila* ADH will offer the possibility to analyze the mechanism of catalysis, to define the different protein domains and residues involved in catalytic activity, and to approach the evolutionary dynamics with respect to the short-chain family and the medium-chain enzymes.

Experimental

Enzyme purification

A wild stock of *Drosophila lebanonensis* was grown in large population cages under standard conditions. 100 g (wet weight) of adults were used for each purification.

The main steps of the purification procedure have been described elsewhere, (Juan & Gonzàlez-Duarte, 1980; Ribas de Pouplana *et al.*, 1991). Nevertheless modifications have been introduced gradually to improve the quality and yield of the purified enzyme. Thus, the previous Blue-Sepharose step was performed with Blue-Sepharose 6 Fast Flow as a gel matrix, using a shorter column (15 × 2.5 cm) and the resolution of the last step was considerably improved with HR-Sephacryl S-200 (100 × 1 cm) in place of the conventional Sephacryl S-200 which had been used before.

Crystallization and crystal stabilization

A screening for optimal crystallization conditions (Carter & Carter, 1979) was performed by the vapour-diffusion method using a screening system with 48 different conditions in a pH range $4.5 < pH < 9.5$. The protein crystallized from 12 of these conditions. Many different crystal shapes were observed. One optimal crystal form, thick plate-like prisms, (see Fig. 1) were grown at 293 K from sitting droplets using a reservoir solution containing 28% PEG 2000, 0.2 *M* $CaCl_2$, 0.1 *M* Tris–HCl buffer, pH 7.5. The droplets were prepared by mixing 0.005 ml of a 5 mg ml^{-1} protein solution in 20 m*M* Tris–HCl buffer, 1% isopropanol, 2 m*M* DTT, pH 8.6, with 0.005 ml reservoir solution. Single crystals were obtained within several days at 293 K, however, there were too many nucleation sites. It was possible to control nucleation with the same conditions in a cold room at 277 K.

The crystals are relatively stable and grow to a size of 0.3–0.5 mm and showed no inhomogeneities in polarized light. To stabilize the crystals before sealing them in glass capillaries they were treated with 33% PEG 2000, 0.2 *M* $CaCl_2$, 0.1 *M* Tris–HCl, pH 7.5.

For X-ray diffraction measurements the crystals were slowly warmed in a Dewar to 285 K, all X-ray measurements were performed at this temperature.

X-ray diffraction experiments

X-ray diffraction intensity data were measured with a Xentronics area detector installed on a Mac Science rotating-anode generator operated at 45 kV and 90 mA. Graphite-monochromatized Cu $K\alpha$ radiation was used. The crystal-to-detector distance was 100 mm. By an ω scan 720 frames of 0.25° were taken in 120 s each. The raw data were evaluated with the *XENGEN* package on a VAX 4000-60 workstation. To reassess the size of the unit cell and the space group of the *Drosophila* alcohol dehydrogenase crystals, in addition photographic exposures were taken on a Huber rotation/precession camera. Rotation photographs were performed using CEA X-ray films at a crystal-to-film distance of 100 mm (see Fig. 2). Exposure times were in the range of 5 h °$rotation^{-1}$ (0.4 mm pinhole collimator).

Crystallographic computations

Native reflection intensity data from 720 frames were evaluated with the *XENGEN* package. Merging and scaling, however, was performed with the *PROTEIN* program system (Steigemann, 1974) using scale and relative isotropic temperature factors. The

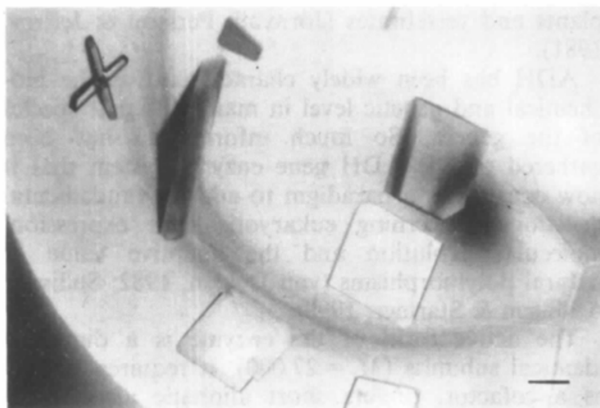

Fig. 1. Monoclinic crystals of *Drosophila lebanonensis* alcohol dehydrogenase grown as described in the experimental section; bar = 0.1 mm.

conversion of the unsorted reflection intensity output from *XENGEN* was performed with the interactive routine *XTP* (program available upon request). *XTP* assigns scaling-group numbers to groups of frames and produces an unmerged output of indices, reflection intensities, σ values, and crystal indentifiers in ascending order of the scaling-group numbers.

A native Patterson map was calculated on a 1.0 Å grid using intensity data ($F^2 > 2\sigma$) in the resolution range 10–4 Å. A self-rotation function (Rossmann & Blow, 1962) was calculated for twofold symmetry axes using the real-space search options of *PROTEIN*. The Patterson space explored was a hollow sphere. Choosing an inner radius limit of 5.0 Å, the origin peak of the map was excluded from the calculations. The outer radius limit was set to 20.0 Å. The results of the calculations were displayed in a ψ, φ, χ polar-angle coordinate system (Rossmann & Blow, 1962) by using a stereogram plot (see Fig. 3).

Results and discussion

Single crystals were obtained within several days at 293 and 277 K (Fig. 1). They had the shape of plate-like prisms and grow to a size of about 0.5 mm in their maximum dimension. The crystals are mechanically relatively stable and insensitive to changes in temperature as well as exposure to X-rays. They diffracted X-rays to beyond 2.3 Å resolution.

According to the symmetry of the reciprocal lattice and the systematic extinctions along the lattice axis $0k0$, these crystals belong to the monoclinic space group $P2_1$. The unit-cell constants of the native crystals are $a = 65.25$, $b = 55.77$, $c = 70.02$ Å, $\alpha = 90$, $\beta = 107.08$, $\gamma = 90°$.

The unit-cell parameters were determined from rotation photographs as well as from a complete native intensity data set to 2.3 Å resolution by autoindexing, linear and non-linear refinement in *XENGEN*.

Biochemical data suggest that the protein is a dimer with the molecular mass 54 kDa composed of two identical subunits (Winberg *et al.*, 1986) under the conditions of a gel-filtration experiment. The size of the unit cell is compatible with the presence of a subunit dimer in the asymmetric unit of the cell, which corresponds to a packing density of 2.15 $Å^3\ Da^{-1}$. This value corresponds very well to the most probable packing densities found in protein crystals (Matthews, 1968).

Diffraction data of one native crystal were collected with the Xentronics area detector and processed with the *XENGEN* software. Reflections were merged and loaded with *PROTEIN* (Steigemann, 1974). From 27 432 measurements 14 628 were unique with a linear R factor of the mean values of the scaled intensities of 0.08. The completeness of the

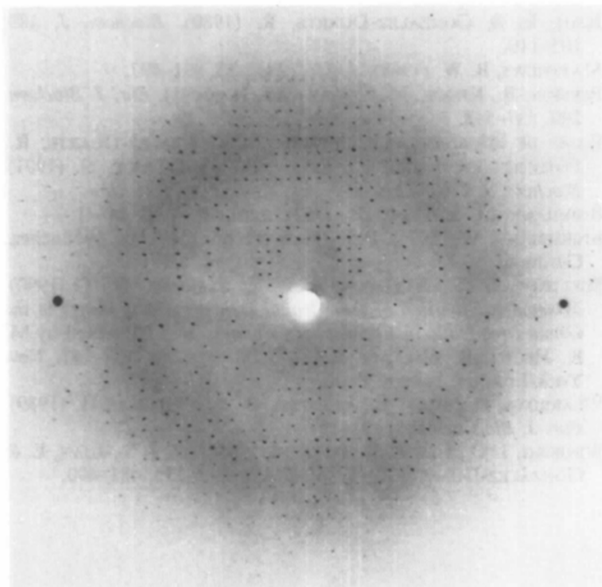

Fig. 2. Screenless rotation photograph, showing the reciprocal lattice planes hkN; rotation of 3° around **a***axis, crystal-to-film distance 100 mm; pin-hole collimator 0.4 mm; graphite-monochromatized Cu $K\alpha$ radiation; rotating-anode generator set to 45 kV, 90 mA, exposure 5 h °rotation^{-1}.

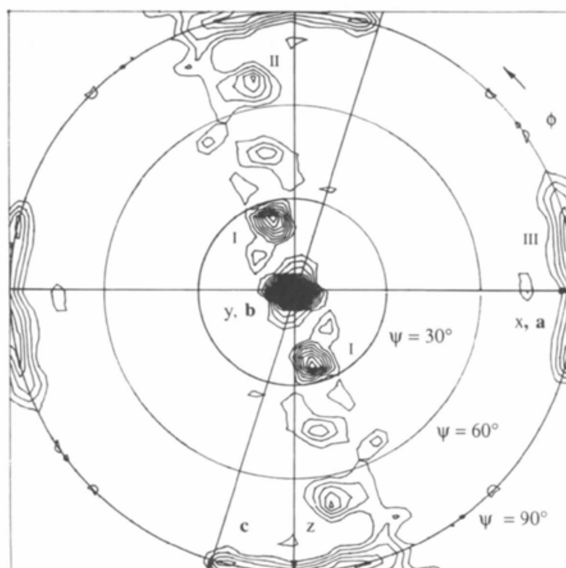

Fig. 3. Stereogram plot of the rotation-function section for twofold local symmetry axes ($\chi = 180°$) derived from correlation calculations in Patterson space. The polar-angle definition follows the convention of Rossmann & Blow (1962). The peak height of the crystallographic twofold axis was set to 100 arbitrary units, mean value: 42.0 (lowest contour line), distance of contour levels: 2 units, $\sigma = 0.6$. Peak I: height = 58.0 units. Peaks II and III: height = 50.0 units.

data was 84% (20–2.5 Å) and 46% in the resolution shell 2.59–2.48 Å.

A self-rotation function calculation for twofold symmetry axes ($\chi = 180^\circ$) showed several correlation maxima which are consistent with the assumption of two dimers sitting at two local twofold symmetry axes in the unit cell (Fig. 3). Both dimers are symmetry related by the crystallographic twofold screw axis 2_1, coincident with the y axis.

The rotation function was explored in spherical polar coordinates in 5 and 1° intervals. The clearest result was obtained with an integration radius of 20 Å which is less than the particle diameter. The final conditions selected for the correlation calculations were that the first Patterson function was represented by all data with $F^2 > 2\sigma$ between 10 and 4 Å resolution. From this map 2420 largest peaks ($P > 2\sigma$) were selected to represent the second Patterson function. The expected peaks (I) for the non-crystallographic twofold symmetry axes for the two subunit dimers at $(\psi, \varphi) = (23, -76^\circ)$ and $(\psi, \varphi) = (23, 104^\circ)$ were higher than 26.6σ above the mean value.

No other peaks were higher than 13.3σ above the mean value. The local twofold axes of the alcohol dehydrogenase dimers deviate by about 23° in ψ from the twofold screw axis, which corresponds to the crystallographic b axis. There are several lower correlation maxima (denoted II and III) present in the stereogram plot which cannot easily be interpreted by twofold local dimer symmetries.

However, there is evidence that *Drosophila* ADH shows structural homology to other short-chain dehydrogenases. The structure of one member of this family, 3α-20β-hydroxysteroid dehydrogenase, is known to high resolution and has revealed a subunit fold, which clearly shows pseudo-twofold symmetry among the secondary-structure elements in one monomer (Ghosh *et al.*, 1991). Thus, additional peaks with lower correlation are possible which can reflect pseudo-222 symmetry in one dimer, *e.g.* the triple of peaks I, II, III which are all 90° apart. Alternatively, these peaks can reflect pseudo-222 symmetry in the vector set created by the two crystallographically related dimers.

The structure analysis of *Drosophila* ADH by crystallographic methods is in progress. In addition to the native data a mercury heavy-atom intensity data set has been collected to 2.5 Å resolution, which could be interpreted in terms of two heavy-atom sites. We are presently trying to solve the structure by Patterson rotation and translation searches with a search model derived from hydroxy steroid dehydrogenase (Ghosh, 1993) and phase combination with single isomorphous replacement phases. The highest solution of the cross-rotation between the search model and the ADH crystal Patterson map correlates well with the maximum at $(\psi, \varphi) = (23, -76^\circ)$ of the alcohol dehydrogenase self rotation, shown in this paper.

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