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Crystallization and preliminary X-ray analysis of substrate complexes of leucine dehydrogenase from *Thermoactinomyces intermedius*

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Leucine dehydrogenase is an octameric enzyme which belongs to the superfamily of amino-acid dehydrogenases and catalyses the reversible oxidative deamination of leucine to 2-ketoisocaproate, with the corresponding reduction of the cofactor NAD^+. Catalysis by this enzyme is thought to involve a large-scale motion of the enzyme's two domains between an 'open' and 'closed' form, with the latter representing a conformation of the enzyme in which the partners involved in the hydride-transfer reaction are appropriately positioned for catalysis. Whilst a structure for the open form of the enzyme has been determined, the nature of the closed form has yet to be observed. In order to trap a closed form, crystals of the complexes of leucine dehydrogenase from *Thermoactinomyces intermedius* with 2-ketoisocaproate and with 2-ketoisocaproate and NAD^+ have been obtained by the hanging-drop vapour-diffusion method using PEG 4000 as a precipitant. The crystals of the binary complex with 2-ketoisocaproate belong to space group $P2_12_12_1$, with approximate unit-cell parameters $a = 106$, $b = 118$, $c = 320$ Å and an octamer in the asymmetric unit, corresponding to a V_M of 3.1 Å^3 Da^{-1}. The crystals of the non-productive ternary complex belong to space group $P6_1$ or $P6_5$, with approximate unit-cell parameters $a = b = 117$, $c = 502$ Å and an octamer in the asymmetric unit, corresponding to a V_M of 3.0 Å^3 Da^{-1}. These crystals diffract X-rays on a synchrotron-radiation source to at least 2.8 and 3.3 Å resolution, respectively, and are suitable for a full structure determination.

1. Introduction

The oxidative deamination of amino acids to their corresponding keto acids is of central importance in metabolism and is catalyzed by the family of amino-acid dehydrogenases. Leucine dehydrogenase (LeuDH; EC 1.4.1.9) catalyses the reversible oxidative deamination of leucine to 2-ketoisocaproate, with the corresponding reduction of the cofactor NAD^+,

$$\text{leucine} + NAD^+ + H_2O \leftrightarrow NADH + NH_4^+ + H^+ + \text{2-ketoisocaproate}.$$

LeuDH was first isolated from *Bacillus cereus* (Sanwal & Zink, 1961) and has been characterized in many *Bacillus* species (Sanwal & Zink, 1962), where it functions in the catabolism of branched-chain amino acids. The preferred substrate is always leucine, but other amino acids such as valine and isoleucine are also accepted by the enzyme (Ohshima *et al.*, 1978). The three-dimensional structure of *B. sphaericus* LeuDH has previously been reported to a resolution of 2.2 Å (Turnbull *et al.*, 1994; Baker *et al.*, 1995). These studies have shown that the enzyme consists of an octameric arrangement of subunits of molecular mass 41 kDa in *D*4 symmetry. The LeuDH subunit is folded into two domains separated by a deep cleft; analysis of a crystal of the binary complex of LeuDH with NAD^+ revealed that this cofactor binds on the surface of one domain with the nicotinamide ring deep in the cleft. This octameric structure of LeuDH is similar to the quaternary structure proposed for a number of phenylalanine dehydrogenases (PheDH; Asano *et al.*, 1987; Pasquo *et al.*, 1998), but contrasts with the dimeric structures proposed for valine dehydrogenase (ValDH; Turnbull *et al.*, 1997) and for some PheDHs (Vanhooke *et al.*, 1999) and the hexameric structure seen in glutamate dehydrogenase (GluDH; Baker *et al.*, 1992). Cloning and sequencing of LeuDH from *T. intermedius* have shown that this enzyme contains 366 amino acids and is homologous to other members of the enzyme superfamily (Ohshima *et al.*, 1994; Galkin *et al.*, 1997).

Structural studies on GluDH, LeuDH and PheDH have suggested that transitions between an 'open' form of the enzyme to which substrates bind and a 'closed' form in which the partners involved in hydride transfer are in a suitable orientation for catalysis are important in this superfamily. For GluDH, the

structures of both open and closed forms of the enzyme have been determined (Baker *et al.*, 1992; Stillman *et al.*, 1993). For LeuDH and PheDH, only an open form and a closed form have been characterized, respectively (Baker *et al.*, 1995; Vanhooke *et al.*, 1999; Brunhuber *et al.*, 2000).

In this paper, we describe the purification, crystallization and preliminary X-ray analysis of LeuDH from *T. intermedius* crystallized as complexes with 2-ketoisocaproate and with NAD^+ and 2-ketoisocaproate. Elucidation of the three-dimensional structures of these complexes and comparison with that of the free enzyme will provide insights into the domain motion thought to be necessary for catalysis.

2. Materials and methods

Transformation of *Escherichia coli* strain TG1 by a plasmid, pLeuDH, carrying the gene for *T. intermedius* LeuDH cloned into (pUC119) was performed as previously described (Galkin *et al.*, 1997) and transformed cells were plated onto LB plates containing 100 µg ml^{-1} ampicillin. A single colony was picked and grown overnight at 310 K in 50 ml LB medium containing 50 µg ml^{-1} ampicillin. The next day, 250 ml of fresh LB medium containing ampicillin (at a final concentration of 50 µg ml^{-1}) was inoculated with 20 ml of the overnight culture. The culture was grown at 310 K to an A_{600} of 0.6 before IPTG was added to a final concentration of 0.5 m*M* to induce expression. The culture was then incubated overnight at 298 K and the cell paste was harvested, frozen in liquid nitrogen and stored at 253 K.

To purify LeuDH, cell paste was defrosted and suspended in buffer *A* (20 m*M* potassium phosphate pH 7.8). Cells were disrupted by sonication at 16 µm amplitude for 4 × 20 s and debris was removed by centrifugation at 40 000*g* for 20 min. The supernatant fraction was applied to a column containing DEAE-Sepharose Fast Flow (Pharmacia) and proteins were eluted with a gradient of NaCl concentration from 0 to 0.5 *M* in buffer *A*. Fractions with the highest specific activity were combined, brought to 2 *M* ammonium sulfate by addition of 4 *M* ammonium sulfate solution and loaded onto a column of Toyopearl Phenyl-650S (Tosohaas, USA). Proteins were eluted from this column by a reverse gradient of ammonium sulfate concentration from 1.8 to 0 *M* in buffer *A*. Fractions with the highest specific activity were combined and concentrated to 2 ml on a VivaSpin concentrator (Viva Science). For further purification, gel filtration was performed on a 1.6 × 60 cm Hi-Load Superdex-200 column (Pharmacia) in 40 m*M* Tris–HCl pH 8.0, 0.15 *M* NaCl. The purity of the LeuDH was estimated by SDS–PAGE to be approximately 95%.

Enzymatic activity (for the oxidative deamination reaction) was measured as previously described (Schutte *et al.*, 1985), but with the reaction carried out at room temperature rather than 323 K and using 1 ml of reaction mixture instead of 3 ml. The protein concentration was estimated by the method of Bradford (1976) using a Bio-Rad Dye Reagent with bovine albumin as a standard. NAD^+ and 2-ketoisocaproate were purchased from Sigma.

For the formation of LeuDH complexes, a 12 mg ml^{-1} solution of LeuDH in 20 m*M* Tris–HCl pH 7.8 with 50 m*M* NaCl was incubated with 20 m*M* 2-ketoisocaproate or with 5 m*M* NAD^+ and 20 m*M* 2-ketoisocaproate for about 1 h at room temperature. Crystallization was achieved using the hanging-drop vapour-diffusion technique by mixing 1–2 µl of the above protein complexes with an equal volume of precipitant and equilibrating over the precipitant well at 290 K. Initial crystals of both complexes were obtained from the Crystal Screen 1 crystallization kit (Hampton Research) condition No. 37 (8% PEG 4000, 0.1 *M* sodium acetate trihydrate pH 4.6). After optimization of the crystallization conditions, cube-like crystals of the complex of LeuDH with 2-ketoisocaproate with a maximum dimension of 0.2 mm were grown at a protein concentration of 4 mg ml^{-1} from 5% PEG 4000 in 0.1 *M* sodium acetate pH 4.8 in 48 h. Hexagonal pyramid-shaped crystals of the non-productive ternary complex of LeuDH with NAD^+ and 2-ketoisocaproate with a maximum dimension of 0.6 mm were grown at a protein concentration of 6–8 mg ml^{-1} from 1.2–3.5% PEG 4000 in 0.1 *M* sodium acetate pH 4.6.

To perform data collection at cryogenic temperatures, the crystals were soaked in a solution containing a mother liquor based on the optimized crystallization conditions with an additional 20% glycerol and flash-frozen in a stream of nitrogen gas at 100 K using an Oxford Cryosystems Cryostream device. X-ray diffraction data for the binary complex of LeuDH with 2-ketoisocaproate were collected by the rotation method with 0.5° rotation per frame at an X-ray wavelength of 0.87 Å on station 9.6 at the CCLRC Synchrotron Source, Daresbury using an ADSC Q4 detector. X-ray diffraction data for the complex of LeuDH with NAD^+ and 2-ketoisocaproate were collected by the rotation method with 0.35° rotation per frame at an X-ray wavelength of 1.488 Å on station 14.1 at the CCLRC Synchrotron Source, Daresbury using an ADSC Q4 detector. The data were processed using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997) and subsequently handled with the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Crystallization of the binary complex of LeuDH with 2-ketoisocaproate and of the non-productive ternary complex of LeuDH with NAD^+ and 2-ketoisocaproate was achieved using the hanging-drop vapour-diffusion technique (Davies & Segal, 1971). Whilst initial crystals of both complexes were obtained using the same conditions, subsequent optimization showed that the optimal protein concentration and PEG concentration for crystallization of the binary and ternary complexes are different. Thus, cube-like crystals of the binary complex of LeuDH with 2-ketoisocaproate were grown at a lower protein concentration than the hexagonal pyramid-shaped crystals of the ternary complex of LeuDH with NAD^+ and 2-ketoisocaproate (4 and 6–8 mg ml^{-1}, respectively), but at a higher PEG concentration (5 and 1.2–3.5% PEG 4000, respectively).

Analysis of a preliminary data set collected on a LeuDH/2-ketoisocaproate binary complex crystal with the auto-indexing routine in *DENZO* (Otwinowski & Minor, 1997) is consistent with a primitive crystal system, class 222, with unit-cell parameters $a = 106.2$, $b = 118.1$, $c = 319.7$ Å. Analysis of systematic absences along the axes suggests that the crystals belong to the space group $P2_12_12_1$. Taking the subunit molecular weight to be 41 kDa, this would suggest that the crystals contain an octamer in the asymmetric unit with a V_M of 3.1 $Å^3$ Da^{-1}, which is within the range given by Matthews (1977). Significant reflections were observed beyond 2.8 Å resolution and a data set to this resolution was collected with the average $I/\sigma(I)$ value being 13.4 for all reflections and 2.3 in the highest resolution shell (2.8–2.83 Å). A total of 336 313 measurements were made of 80 302 independent reflections. Data processing gave a data set that is 81% complete with an R_{merge} of 0.099 for intensities (0.238 in the highest resolution shell).

Analysis of a preliminary data set collected on the LeuDH/NAD^+/2-ketoiso-

caproate non-productive ternary complex with the autoindexing routine in *DENZO* (Otwinowski & Minor, 1997) showed that the crystals of the complex belong to the primitive crystal system, class 6 or 62, with unit-cell parameters $a = b = 116.6$, $c = 502.5$ Å. Analysis of systematic absences along the l axis shows that this axis is either a 6_1 or 6_5 screw axis. Attempts to merge this data set in the *SCALEPACK* package (Otwinowski & Minor, 1997) has excluded a primitive crystal system class 62 from consideration owing to an extremely high $R_{\rm merge}$. Thus, the space group would appear to be either $P6_1$ or $P6_5$. Taking the subunit molecular weight to be 41 kDa, this would suggest that the crystals contain an octamer in the asymmetric unit with a $V_{\rm M}$ of 3.0 Å^3 Da^{-1}, which is within the range given by Matthews (1977). Significant reflections were observed beyond 3.3 Å resolution and a data set to this resolution was collected, with the average $I/\sigma(I)$ value being 11.5 for all reflections and 3.4 in the highest resolution shell (3.3–3.38 Å). A total of 179 141 measurements were made of 52 595 independent reflections. Data processing gave a data set that is 91.5% complete with an $R_{\rm merge}$ of 0.107 for intensities (0.245 in the highest resolution shell).

A self-rotation function of the LeuDH non-productive ternary complex data set was calculated with the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) using data in the resolution range 15–5 Å and a 50 Å radius of integration. The presence of a peak in the $\kappa = 90^\circ$ section at $\omega = 0^\circ$ (Fig. 1*a*) and peaks in the $\kappa = 180^\circ$ section at $\omega = 90^\circ$ and every 15° in φ (Fig. 1*b*) is consistent with a particle with $D4$ symmetry with a non-crystallographic fourfold axis parallel to the crystallographic sixfold axis and with non-crystallographic twofold axes lying in the *ab* plane.

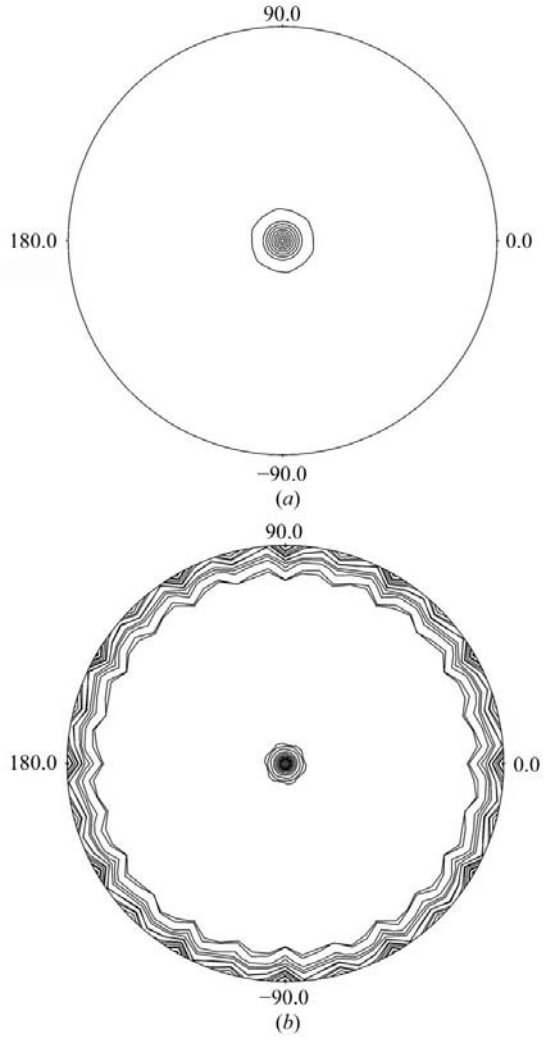

Figure 1
A projection of (*a*) the $\kappa = 90^\circ$ and (*b*) the $\kappa = 180^\circ$ sections of the self-rotation function of the *T. intermedius* LeuDH non-productive ternary complex data set calculated using data in the resolution range 15–5 Å and with a 50 Å radius of integration.

A preliminary attempt has been made to solve the structures of these LeuDH complexes by molecular-replacement techniques using the fast direct rotation search as implemented in *CNS* (Brünger, 1998and a search model for the octamer derived from the structure of native *B. sphaericus* LeuDH (PDB code 1leh; Baker *et al.*, 1995). Using data in the 15.0–4.0 Å resolution range for the crystals of the LeuDH binary complex with 2-ketoisocaproate, the cross-rotation function gave a clear solution with a peak height of 4σ. A subsequent translation-function search gave a model which refined to an R factor of 44% after rigid-body refinement. Analysis of the real-space R factor for this solution revealed a good correlation for residues 1–143 and 331–363, but a poor correlation for the remaining part of the structure, residues 144–330 (Fig. 2). In this enzyme superfamily the enzymes fold into two domains, which are known to undergo a large domain closure on substrate binding as previously shown in GluDH (Stillman *et al.*, 1993). Since the search model is that of an open form of the enzyme this is consistent with the structure trapped in their crystal form being that of a closed form. However, further refinement will be necessary to confirm these findings. Equivalent results were seen for the crystal of the ternary complex (data not shown).

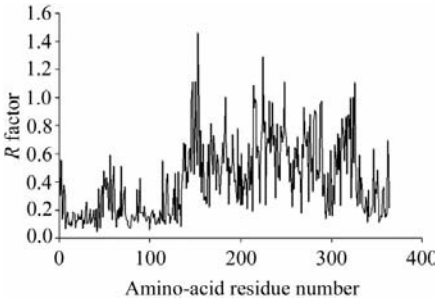

Figure 2
The real-space R factor for one monomer of the binary complex of *T. intermedius* LeuDH with 2-ketoisocaproate after preliminary refinement.

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