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Facile crystallization of *Escherichia coli* ketol-acid reductoisomerase

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Ketol-acid reductoisomerase (EC 1.1.1.86) catalyses the second reaction in the biosynthesis of branched-chain amino acids. The reaction involves an Mg^{2+}-dependent alkyl migration followed by an NADPH-dependent reduction of the 2-keto group. Here, the crystallization of the *Escherichia coli* enzyme is reported. A form with a C-terminal hexahistidine tag could be crystallized under 18 different conditions in the absence of NADPH or Mg^{2+} and a further six crystallization conditions were identified with one or both ligands. With the hexahistidine tag on the N-terminus, 20 crystallization conditions were found, some of which required the presence of NADPH, $NADP^+$, Mg^{2+} or a combination of ligands. Finally, the selenomethionine-substituted enzyme with the N-terminal tag crystallized under 15 conditions. Thus, the enzyme is remarkably easy to crystallize. Most of the crystals diffract poorly but several data sets were collected at better than 3.2 Å resolution; attempts to phase them are currently in progress.

1. Introduction

Ketol-acid reductoisomerase (KARI; EC 1.1.1.86; reviewed by Dumas *et al.*, 2001) catalyzes the second reaction in the biosynthesis of branched-chain amino acids, converting either 2-hydroxy-3-keto-2-methylbutyrate (acetolactate) or 2-hydroxy-3-keto-2-ethylbutyrate to the corresponding 2,3-dihydroxy-3-alkylbutyrate. The reaction occurs in two stages (Chunduru *et al.*, 1989; Dumas *et al.*, 1995) involving an Mg^{2+}-dependent alkyl migration to form a 3-hydroxy-2-keto-3-alkylbutyrate intermediate followed by an Mg^{2+}-, Mn^{2+}- or Co^{2+}-dependent reduction of the 2-keto function by NADPH. Since the biosynthetic pathway is absent in animals, but present in microorganisms and plants, KARI is a potential target for both antimicrobial compounds and herbicides. Inhibitors have been developed (Schulz *et al.*, 1988; Aulabaugh & Schloss, 1990) as possible herbicides but have not proved to be very active towards plants (Dumas *et al.*, 1994).

Ahn *et al.* (2003) have grouped KARI proteins into two classes: class I enzymes contain 330–340 amino-acid residues, while class II have lengths of ~490 residues because of an extra segment near the middle of the protein. Class I KARIs are typically found in microorganisms, while class II KARIs are characteristic of plants. However, there are scattered exceptions amongst the γ-proteobacteria, including *Escherichia coli*, which contains a class II KARI (Ahn *et al.*, 2003).

Three-dimensional structures of both class I (*Pseudomonas aeruginosa*; Ahn *et al.*, 2003) and class II (spinach; Biou *et al.*, 1997; Thomazeau *et al.*, 2000) KARIs have been published. The middle and C-terminal domains of spinach KARI spatially overlap the C-terminal domains of a pair of *P. aeruginosa* KARI monomers. As a result, the active site of spinach KARI is contained entirely within a monomer, whilst that of *P. aeruginosa* KARI is made up of residues from two monomers. Notwithstanding this radically different organization, almost all active-site residues are conserved spatially.

The three-dimensional structure of class II bacterial KARI has yet to be solved and we have therefore attempted to crystallize the *E. coli* enzyme. The protein proved to be remarkably easy to crystallize and a variety of distinct forms were obtained readily.

2. Materials and methods

2.1. Materials

Materials were purchased from the following common commercial suppliers. Crystal Screens (1, 2, Lite, Ammonium Sulfate, PEG/LiCl, PEG 6000 and Natrix) were from Hampton Research and Wizard crystal screens were from Emerald Biostructures.

2.2. Protein expression and purification

E. coli KARI with an N-terminal hexahistidine tag, hereafter denoted N-KARI, was expressed as described previously (Hill & Duggleby, 1999). To express the protein with a C-terminal hexahistidine tag (C-KARI), a new

construct was developed in which the protein has a native N-terminus and a C-terminus to which is added the sequence LEHHHHHH. To obtain the selenomethionine-substituted protein, expression was performed by a method similar to that described by Thomazeau *et al.* (2001). The enzymes were purified as described previously and stored at 203 K in 20 m*M* Na HEPES buffer pH 7.5, except that for SeMet KARI purification buffers were degassed just prior to purification, fractions (4 ml) were collected in tubes containing 1 µl 1 *M* DTT and the enzyme was stored in buffer supplemented with 1 m*M* DTT and 0.2 m*M* Na EDTA.

2.3. Crystallization

Crystals were obtained using the hanging-drop vapour-diffusion method. Various crystallization conditions were initially identified using commercial screens. Trials were carried out at 291 K in 24-well plates containing 500 µl screen reagent and drops formed by mixing 3 µl each of well reagent and protein (5–15 mg ml^{-1}).

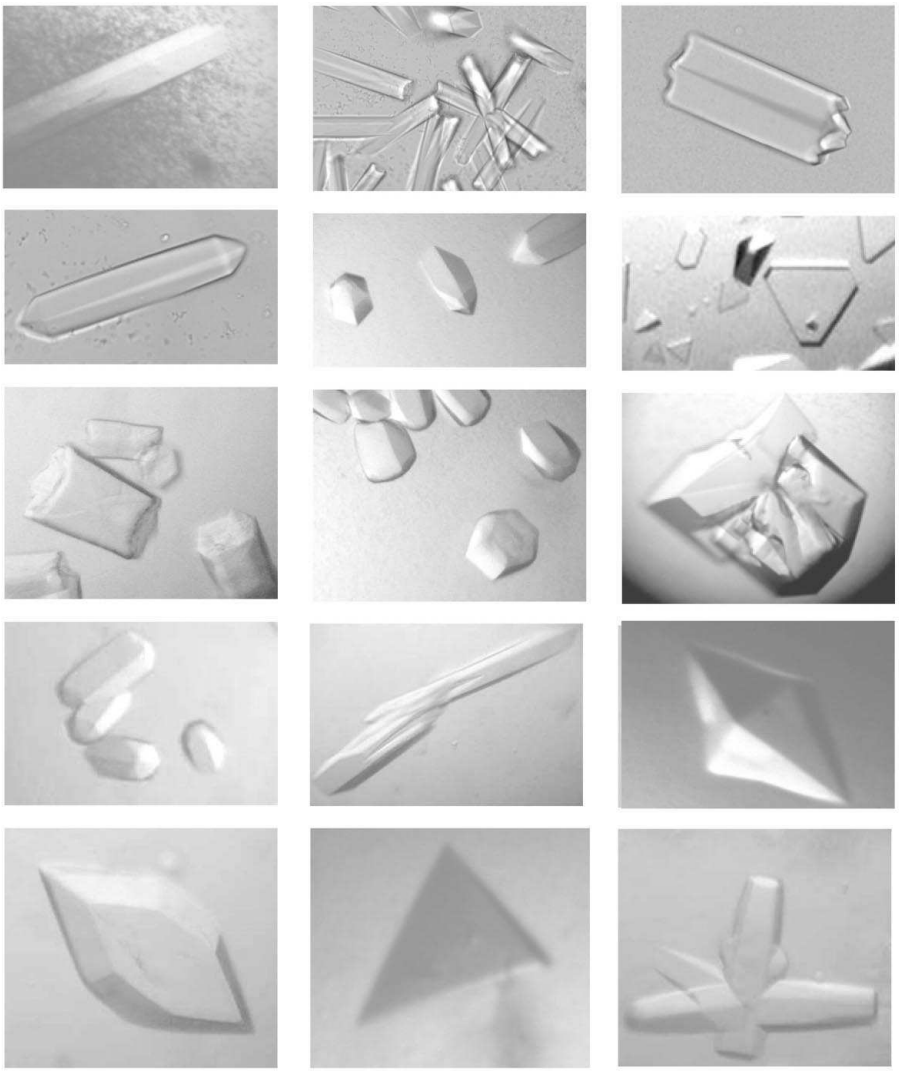

Figure 1
Crystals obtained under various conditions reported in supplementary material Table *A*. Row 1, left to right: conditions 4 [C-KARI with 5%(*w*/*v*) PEG 6000, 0.1 *M* sodium citrate pH 5.0], 6 [C-KARI with 10%(*v*/*v*) isopropanol, 10%(*w*/*v*) PEG 4000, 0.1 *M* sodium citrate pH 5.6] and 19 [C-KARI with 10%(*w*/*v*) PEG 3000, 0.2 *M* $MgCl_2$, 0.1 *M* sodium cacodylate pH 6.5]; row 2, 16 [C-KARI with 20%(*w*/*v*) PEG 6000, 0.1 *M* Na Bicine pH 9.0], 20 [C-KARI with 20%(*w*/*v*) PEG 1000, 0.2 *M* $MgCl_2$, 0.1 *M* sodium cacodylate pH 6.5] and 54 [SeMet-N-KARI with 15%(*w*/*v*) PEG 4000, 0.2 *M* Li_2SO_4, 0.1 *M* Tris–HCl pH 8.5]; row 3, 22 [C-KARI with NADPH, 10%(*w*/*v*) PEG 8000, 0.2 *M* NaCl, 0.1 *M* sodium/potassium phosphate pH 6.2], 24 [C-KARI with NADPH, 10%(*w*/*v*) PEG 3000, 0.2 *M* $MgCl_2$, 0.1 *M* sodium cacodylate pH 6.5] and 35 [N-KARI with NADPH, 30%(*v*/*v*) PEG MME 550, 0.1 *M* NaCl, 0.1 *M* Na Bicine pH 9.0]; row 4, 23 [C-KARI with NADPH, 1.6 *M* $(NH_4)_2SO_4$, 0.1 *M* Na Bicine pH 9.0], 26 [N-KARI with 10%(*w*/*v*) PEG 6000, 4.8%(*v*/*v*) MPD, 0.1 *M* Na HEPES pH 7.5] and 33 [N-KARI with NADPH, 0.68 *M* trisodium citrate, 0.1 *M* Tris–HCl pH 8.5]; row 5, 27 [N-KARI with 15%(*w*/*v*) PEG 4000, 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5], 45 [SeMet-N-KARI with 20%(*w*/*v*) PEG 3000, 0.1 *M* sodium citrate pH 5.5] and 55 [SeMet-N-KARI with 15%(*w*/*v*) PEG 4000, 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5].

2.4. X-ray data collection

All the KARI crystals were mounted either in a quartz capillary tube for room-temperature data collection or in a nylon loop that was cryocooled in a liquid-nitrogen stream (Oxford Cryosystems Cryostream). For cryocooling, the crystals were transferred to an appropriate solution that varied for different crystals. Before freezing, some crystals were dehydrated by placing the drop containing the crystals over a reservoir with progressively higher (20–30% in steps of 5%) precipitant concentrations (Heras *et al.*, 2003).

For the N-KARI crystals, preliminary screening of crystals was performed using an R-AXIS IV^{++} image-plate detector and a Rigaku RU-200 generator at the University of Queensland. Data sets were collected at ESRF (Grenoble, France) on beamlines BM14 and ID14-3 and at the Advanced Photon Source, Argonne National Laboratory, Chicago, USA with an ADSC Q4 CCD detector on beamlines 14ID-B (MAD) or 14ID-D (all other data). The N-KARI data were integrated with *DENZO* and scaled and merged with *SCALEPACK* (Otwinowski & Minor, 1997). A complete data set for C-KARI in complex with NADPH was collected using an FR-E X-ray generator operated at 45 kV and 45 mA and an R-AXIS IV^{++} image-plate area detector at the University of Queensland. The images were scaled and merged using the *CrystalClear* v.1.3.5 software (Rigaku/MSC, Texas, USA; Pflugrath, 1999).

3. Results and discussion

3.1. Protein expression and purification

The specific activity of C-KARI (1.2 U mg^{-1}) is comparable to that of N-KARI (1.5 U mg^{-1}). Selenomethionine-substituted N-KARI has a specific activity of 1.3 U mg^{-1} and the incorporation of selenomethionine was 91% as determined by amino-acid analysis.

3.2. Crystallization

E. coli KARI proved to be exceptionally easy to crystallize using commercial formulations. Some formulations were varied to improve the crystals. C-KARI crystallized under 18 different conditions in the absence of added active-site ligands and three formulations that contained the Mg^{2+} cation cofactor (see Table *A* in the supplementary

Table 1
Diffraction data for *E. coli* KARI.

Values in parentheses are statistics for the outer resolution shell: 2.69–2.60 Å for condition 23 [C-KARI with NADPH, 1.6 *M* $(NH_4)_2SO_4$, 0.1 *M* Na Bicine pH 9.0], 3.0–2.9 Å for condition 27 [N-KARI with 15%(*w*/*v*) PEG 4000, 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5] and 3.30–3.15 Å for condition 55 [SeMet-N-KARI with 15%(*w*/*v*) PEG 4000, 0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5].

Condition	23	27	55
Temperature (K)	100	100	100
Resolution (Å)	32.48–2.60	100–2.90	100–3.15
No. observations	922387 (62352)	166739 (6902)	352826 (5685)
Unique reflections	106858 (10489)	54141 (3441)	43994 (3155)
Completeness (%)	99.8 (98.5)	89.3 (57.5)	91.4 (47.4)
$\langle I/\sigma(I)\rangle$	20.9 (5.1)	11.3 (6.0)	7.1 (2.5)
R_{sym}†	0.074 (0.300)	0.099 (0.187)	0.088 (0.153)

† $R_{sym} = \sum|I - \langle I\rangle|/\sum\langle I\rangle$.

material). Crystals of N-KARI were obtained with no added ligands, with added Mg^{2+}, NADPH or $NADP^+$ and with combinations of these ligands (see Table *A* in supplementary material). SeMet-N-KARI also crystallized under a range of conditions (see Table *A* in the supplementary material); no trials were undertaken with ligands, except for Mg^{2+}, which is present in two of the formulations. Some of the crystals obtained are illustrated in Fig. 1.

Paradoxically, although crystals were obtained easily, conditions that yielded high-resolution diffraction data were difficult to find. There was a bewildering array of starting conditions to choose for optimization and many of these led to crystals with an attractive appearance but that diffracted to only 4 Å resolution or weaker. The crystals showed a wide range of unit-cell parameters and space groups (see Table *B* in the supplementary material). Reflection data collected to better than 3.2 Å were obtained from crystals grown under conditions 23, 27 and 55 (see Table 1). For SeMet-N-KARI (condition 55), we commenced collection of a MAD data set, but degradation of the crystal allowed only limited information to be obtained at the third wavelength.

3.3. Diffraction data

Two data sets for KARI have been obtained at better than 3.0 Å resolution, with the best being the C-KARI crystals obtained from condition 23, which diffract to 2.6 Å resolution. On the basis that the Matthews coefficient is in the normal range for proteins (Matthews, 1968), there appear to be six monomers in the asymmetric unit. Attempts to phase data sets by molecular replacement with *CNS* (Brünger *et al.*, 1998) and *AMoRe* (Navaza, 1994) using various models based on parts of spinach or *P. aeruginosa* KARI failed to identify the correct solutions. Using the data obtained for the best selenomethionine-substituted N-KARI crystals, the programs *CNS* (Brünger *et al.*, 1998), *SnB* (Weeks & Miller, 1999) and *SOLVE* (Terwilliger & Berendzen, 1999) have not been able to clearly identify the locations of the Se atoms. New attempts at molecular replacement using the program *EPMR* (Kissinger *et al.*, 1999) are now under way.

4. Conclusions

KARI is an easy protein to crystallize. Unfortunately, the resolution of data obtained for the majority of these crystals is worse than 3.0 Å, suggesting that while there are some good contacts leading to crystallization, there are insufficient contacts to highly order the majority of the protein and tag atoms in these crystals. Once the crystal structures begin to emerge, *E. coli* KARI will provide an interesting case study to observe the structural features that lead to its ease of crystallization.

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[1] Supplementary material has been deposited in the IUCr electronic archive (Reference TS5013). Details for accessing these data are described at the back of the journal.

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