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Crystallization of 5-keto-4-deoxyuronate isomerase from *Escherichia coli*

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Abstract

5-keto-4-deoxyuronate isomerase from *Escherichia coli* has been crystallized after partial purification. The isomerase was found to be enriched in preparations of an unrelated recombinant protein. Crystals of the isomerase were obtained from two different precipitants despite the fact that the recombinant protein represented roughly 90% of the total protein present. The crystals diffract to 2.7 Å resolution and are suitable for a structure determination. The role of the isomerase in *E. coli* is uncertain, as *E. coli* is not known to degrade the polysaccharides which are potential sources of 5-keto-4-deoxyuronate.

1. Introduction

The function and regulation of many of the gene products of *E. coli* are unknown. We report that the enzyme 5-keto-4-deoxyuronate isomerase (Priess & Ashwell, 1963) can be partially purified and crystallized from a soluble extract of *E. coli* grown in a complete medium. Although the structure of the enzyme has not been characterized, its genetics and regulation have been studied in *Erwinia chrysanthemi* (Condemine & Robert-Baudouy, 1991; Nasser *et al.*, 1994; Reverchon *et al.*, 1991). In *E. chrysanthemi*, 5-keto-4-deoxyuronate isomerase is one of the enzymes that converts unsaturated hexuronic acids, formed upon the breakdown of pectin by lyases, to 2-keto-3-deoxygluconate for subsequent metabolism (Reverchon *et al.*, 1991). Pectin is a major polysaccharide in plant cell walls, and the breakdown of pectin is believed to be partially responsible for the soft root rot observed when *E. chrysanthemi* infects certain plants (Collmer & Keen, 1986). The observation reported here that *E. coli* expresses 5-keto-4-deoxyuronate isomerase raises questions about the source of 5-keto-4-deoxyuronate and the metabolism of unsaturated hexuronic acids in *E. coli*.

2. Methods

2.1. *Protein expression and purification*

The purification methods for 5-keto-4-deoxyuronate isomerase were initially developed for the purification of the *Rhodobacter capsulatus S*-adenosylhomocysteine (AdoHcy) hydrolase (E.C. 3.3.1.1). The growth of *E. coli* XL1-Blue (Stratagene) expressing the recombinant *R. capsulatus* AdoHcy hydrolase and purification of the enzyme followed the methods used to purify the recombinant rat AdoHcy hydrolase from *E. coli* (Aksamit *et al.*, 1994; Gomi *et al.*, 1989; Sganga *et al.*, 1992). Bacteria were cultured at 310 K in NZCYM medium containing 100 μg ml^{-1} ampicillin, and expression of the AdoHcy hydrolase was induced by adding isopropyl-β-D-thiogalactopyranoside to 1 m*M*. Cells were harvested and resuspended in 25 m*M* Tris (pH 8.0), 50 m*M* glucose, 10 m*M* EDTA containing 5 mg ml^{-1} lysozyme. After incubation with lysozyme for 10 min at room temperature, cells were pelleted and frozen at 203 K.

A crude cell lysate was obtained by thawing the frozen pellet in 20 m*M* Tris (pH 7.4) 7 m*M* β-mercaptoethanol, 0.2 m*M* phenylmethylsulfonyl fluoride. The temperature during the purification was maintained at 273–277 K. Particulate material was removed by centrifugation at 100 000*g* for 1 h. Ammonium sulfate [22.7 g (100 ml)$^{-1}$] was added to the clarified extract, and precipitated proteins were removed by centrifugation. The ammonium sulfate concentration in the supernatant was raised by adding 12.4 g ammonium sulfate per 100 ml, and precipitated proteins were again collected by centrifugation. The pellet from this second ammonium sulfate precipitation was redissolved in 80 m*M* potassium phosphate (pH 7.4), 1 m*M* EDTA (buffer *A*) and dialyzed against buffer *A* overnight. The protein solution was applied to a column of DEAE–Sepharose equilibrated in buffer *A*, and the column was washed with buffer *A*. AdoHcy hydrolase was eluted from the column with a linear salt gradient (0–0.2 *M* KCl in buffer *A*). Column fractions containing enzyme activity (assayed as in Palmer & Abeles, 1979) were pooled, and the pool was concentrated using an Amicon ultrafiltration cell and YM30 membrane. The DEAE pool was applied to a column of Sephacryl S200HR equilibrated in 50 m*M* potassium phosphate (pH 7.2), 1 m*M* EDTA. Fractions containing AdoHcy hydrolase activity were pooled and concentrated by Amicon ultrafiltration to 10 mg protein per ml.

2.2. *Crystallization and data collection*

Crystals for X-ray data collection were grown at room temperature from hanging drops initially containing 3 μl each of precipitant and protein at 10 mg ml^{-1}. The precipitant was 1.5–2.0 *M* ammonium sulfate, 0.1 *M* MES (pH 6.5), with the largest crystals approximately 0.16 × 0.16 × 0.12 mm in size appearing in drops equilibrated against 1.6 *M* ammonium sulfate. In order to screen crystallization conditions including various divalent cations, the phosphate concentration in the protein stock solution was reduced to 0.5 m*M* by successive cycles of dilution and concentration in an Amicon Centricon-30 microconcentrator. With protein in low-phosphate buffer,

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crystals of apparently identical morphology could be obtained from hanging drops when the precipitant was 24–32% PEG 400, 0.2 *M* $CaCl_2$, 0.1 *M* MES (pH 6.5). The crystals grown from PEG 400 were shown to contain the same protein as the crystals grown from ammonium sulfate by sodium dodecyl sulfate (SDS) gel electrophoresis of single crystals dissolved in SDS sample buffer. The diffraction characteristics of the crystals grown in PEG 400 have not been determined, as the crystals tend to be smaller than those obtained from ammonium sulfate. For X-ray data collection, crystals grown in ammonium sulfate were transferred through a series of stabilizing solutions in order to increase the ammonium sulfate concentration to a final value of 1.9 *M*. If the stabilizing step was omitted, a twinned diffraction pattern was observed despite the fact that the crystals appeared to be well formed and grew as single crystals. A room-temperature native data set extending to 3.1 Å resolution was collected from a single crystal using Cu $K\alpha$ radiation from a rotating-anode source and an R-Axis IIc image-plate detector. A cryo-temperature data set was collected from a crystal stabilized in the above fashion and then soaked in a cryoprotectant for 30 min before freezing in a stream of nitrogen at 113 K. The cryoprotectant was 30% glycerol in 1.9 *M* ammonium sulfate, 0.1 *M* MES (pH 6.5). The cryo-temperature data were collected with Cu $K\alpha$ radiation provided by a Rigaku rotating-anode generator equipped with focusing optics and an R-Axis IIc imaging plate. All data sets were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1996).

2.3. *Protein and peptide sequencing*

Two Coomassie-stained gel bands of the 33 kDa component were excised and subjected to *in situ* proteolytic digestion with 1.16 µg modified trypsin (sequencing grade, Promega, Madison, WI) essentially according tor the method of Moritz *et al.* (1995). Washing steps were performed at 323 K. The resulting digest was separated at 0.25 ml min^{-1} with a gradient described by Fernandez *et al.* (1992) on a narrow bore (2.1 × 250 mm) Vydac 218TP52 column and guard column (Separations Group, Hesperia, CA) at 308 K using a System Gold HPLC equipped with a Model 507 autosample, Model 126 programmable solvent module and Model 168 diode array detector (Beckman, Fuller, CA). Column effluent was monitored at 215 and 280 nm. Fractions were collected at 30 s intervals and stored at 203 K.

Protein solutions or fractions (125 µl) containing tryptic peptides were applied in 30 µl aliquots to a Biobrene (Applied Biosystems, Foster City, CA) treated glass fiber filter and dried prior to amino-acid sequencing. Protein solutions were alternatively converted to PVDF blots using ProSpin or ProSorb devices (Applied Biosystems) according to the manufacturer's instructions prior to amino-acid sequencing. Amino-acid sequencing was performed on a Model 477A pulsed-liquid protein sequencer equipped with a Model 120A PTH analyzer and Model 610A data analysis system (Applied Biosystems).

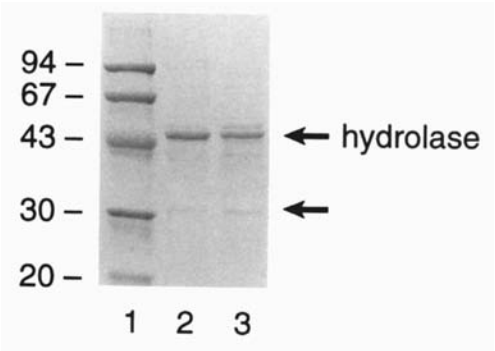

Fig. 1. Preparations of *R. capsulatus* AdoHcy hydrolase which yielded crystals of the 33 kDa *E. coli* kduI protein. Purified preparations of the hydrolase were subjected to SDS polyacrylamide gel electrophoresis and stained with Coomassie blue. Lane 1, low molecular weight calibration kit markers (Pharmacia); lanes 2 and 3, two different preparations of *R. capsulatus* AdoHcy hydrolase. The position of the 51 kDa AdoHcy hydrolase monomer is indicated. The arrow below the AdoHcy hydrolase monomer points to the 33 kDa component.

3. Results

Crystallization trials with a recombinant protein expressed in *E. coli* yielded crystals after 2–4 d in drops initially containing equal volumes of protein at 10 mg ml^{-1} and either ammonium sulfate or PEG 400 as precipitant. The molecular weight of the protein present in the crystals was 33 kDa, as shown by SDS gel electrophoresis of a single crystal dissolved in SDS sample buffer. Surprisingly, the crystals grow from a component of the preparation representing ~10% of the total protein present (Fig. 1). We were able to partially purify the 33 kDa protein from *E. coli* lacking the recombinant plasmid by following the same steps as used in the purification of the recombinant protein. Fractions were analyzed for the presence of the 33 kDa protein on Coomassie-stained SDS polyacrylamide gels. The pooled fractions from the final purification column were concentrated to 1.1 mg protein ml^{-1}, and this material was used in crystallization trials with ammonium sulfate as precipitant. Crystals appeared after 1 d (Fig. 2).

To identify the protein which crystallized, amino-terminal sequence was obtained from the 33 kDa component after SDS gel electrophoresis and transfer of the proteins to PVDF membrane. The sequence at the amino-terminus (MDVRQSIHSAHAK*X*LD*XX*GL) matches that of open reading frame ORF_f278 in *E. coli* (GenBank accession U29581). To confirm the identification, sequence was obtained from an internal tryptic peptide. The internal sequence (RHIVMHNEQAVISPSWSIHSG) corresponds perfectly to residues 227–247 of the *E. coli* open reading frame ORF_f278.

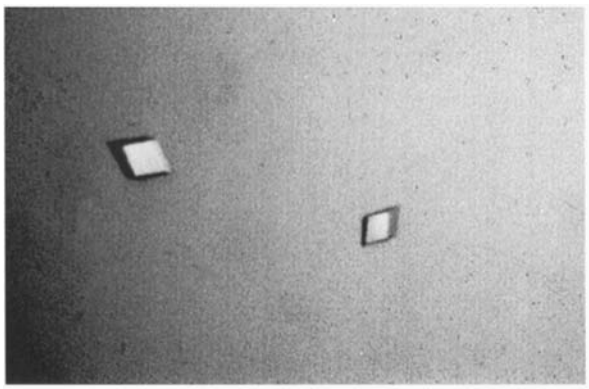

Fig. 2. Crystals of the *E. coli* kduI protein grown from 1.5 *M* ammonium sulfate, 0.1 *M* MES (pH 6.5).

ORF_f278 appears to be the *E. coli* homolog of the *kduI* gene in *Erwinia chrysanthemi* (Condemine & Robert-Baudouy, 1991), as the two sequences display 74% identity (86% similarity) over 278 residues. The calculated molecular weight of the *E. coli* kduI protein is 31 076 in reasonable agreement with the experimental value of 33 000 obtained for the crystallized protein by SDS polyacrylamide gel electrophoresis. A hexameric quarternary structure would be consistent with our gel-filtration results, as the kduI protein was found in the same fractions as the recombinant protein of 4 × 51 kDa.

Work on the structure solution by the method of multiple isomorphous replacement is in progress. Analysis of the diffraction pattern of the crystals grown from ammonium sulfate indicates the protein crystallized in space group *R*32 with $a = b = c = 84.8$ Å, $\alpha = \beta = \gamma = 75.2^\circ$ in the rhombohedral setting, or $a = b = 103.4$, $c = 180.5$ Å in the hexagonal setting. Given a molecular weight of 33 kDa for the monomer in the asymmetric unit, a value of 2.78 Å^3 Da^{-1} was calculated for the Matthews coefficient (Matthews, 1968), corresponding to a solvent content of 56%. The resolution limit of the crystals has been improved by freezing the crystals after transfer to a cryoprotectant containing 30% glycerol. The native data are 98.6% complete to 2.7 Å with an $R_{\rm merge}$ of 3.7% for 32 434 measurements of 9820 unique reflections.

4. Discussion

The physiological role of kduI has been studied in the plant pathogen *E. chrysanthemi*, where genetic studies indicate the *kduI* gene product is 5-keto-4-deoxyuronate isomerase (Condemine & Robert-Baudouy, 1991; Reverchon *et al.*, 1991). In *E. chrysanthemi*, 5-keto-4-deoxyuronate is produced during the breakdown of pectin from plant cell walls. *E. chrysanthemi* secretes lyases which cleave pectin *via* a β-elimination mechanism, generating an unsaturated uronic acid derivative at the non-reducing end of the chain. Further breakdown frees the unsaturated sugar 5-keto-4-deoxyuronate. In a similar series of steps, 5-keto-4-deoxyuronate can be produced *via* breakdown of a number of polysaccharides, including hyaluronic acid, dermatan sulfate, chondroitin sulfates and some bacterial polysaccharides (Kiss, 1974). While *E. coli* is not known to degrade such polysaccharides, the breakdown of polysaccharides by other microorganisms in *E. coli*'s environment could provide a source of 5-keto-4-deoxyuronate. It is worth noting that pure cultures of *E. coli* rarely occur outside the laboratory. An additional source of 5-keto-4-deoxyuronate might be turnover and recycling of the bacterium's own polysaccharides.

Crystallographic studies often yield unanticipated results. To our knowledge, this report is the first example of protein crystals providing the first clue to the presence of a previously undocumented metabolic pathway. That the pathway was uncovered in one of the most intensively studied model organisms is all the more surprising.

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