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Purification, crystallization and preliminary X-ray analysis of isocitrate dehydrogenase kinase/phosphatase from *Escherichia coli*

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The *Escherichia coli aceK* gene encodes isocitrate dehydrogenase kinase/phosphatase (EC 2.7.11.5), a bifunctional protein that phosphorylates and dephosphorylates isocitrate dehydrogenase (IDH), resulting in its inactivation and activation, respectively. This reversible (de)phosphorylation directs isocitrate, an intermediate of the citric acid cycle, to either go through the full cycle or to enter the glyoxylate bypass. In the present study, the AceK protein from *E. coli* has been purified and crystallized. Three crystal forms were obtained from very similar crystallization conditions. The crystals belong to space groups $P4_12_12$, $P3_221$ and $P2_12_12_1$ and diffracted X-rays to resolutions of 2.9, 3.0 and 2.7 Å, respectively.

1. Introduction

The study of bacterial protein-phosphorylation systems saw a major advance as a result of the discovery of the phosphorylation of isocitrate dehydrogenase (IDH) of *Escherichia coli*, which regulates its activity (Garnak & Reeves, 1979). The phosphorylation is performed by AceK, a 68 kDa protein which possesses both protein kinase and phosphatase activities, on the Ser113 residue of IDH (Cortay *et al.*, 1988; Klumpp *et al.*, 1988). This was the first report of a serine/threonine phosphorylation system in bacteria. The unphosphorylated and active IDH promotes normal completion of the citric acid cycle, while the phosphorylated and inactive IDH results in the conversion of isocitrate to glyoxylate by isocitrate lyase (Cozzone, 1998). Glyoxylate is further converted to malate, a downstream product of the regular citric acid cycle (Cozzone, 1998). When the bacteria are grown on acetate, IDH is found in its inactive phosphorylated form, thus preventing the full citric acid cycle (Borthwick *et al.*, 1984; LaPorte *et al.*, 1984). When the bacteria are placed in glucose or pyruvate, IDH is dephosphorylated and the citric acid cycle is completed. Such a molecular switch conserves C atoms for bacterial growth, as the glyoxylate bypass skips the emission of two carbon dioxide molecules with a loss of energy generation during the missing steps of the citric acid cycle. While the kinase and phosphatase activities only act on IDH, AceK also demonstrates an ATPase activity that is independent of IDH (LaPorte, 1993).

The function of AceK and its involvement in the regulation of the citric acid cycle and the glyoxylate bypass have been well characterized, but the structural and mechanistic qualities of the protein have remained relatively unknown. Sequence comparison shows no similarity between AceK and the eukaryotic Ser/Thr protein kinases. Therefore, as a distinct bifunctional protein, AceK may possess a novel kinase/phosphatase structural fold and (de)phosphorylation mechanism. Structural studies would enable analysis of AceK on both the macroscale and the microscale. Determination of the crystal structure could identify the domain(s) responsible for kinase, phosphatase and ATPase activities and provide insights into alteration of these activities of AceK. It is currently unknown whether the active site undergoes a conformational change as it switches between kinase and phosphatase activities. Determination of structures 'caught' in both kinase and phosphatase modes would provide information on the how the bifunctionality of AceK is achieved. In this study, we

describe the cloning, expression, purification, crystallization and preliminary X-ray crystallographic analysis of the AceK protein.

2. Materials and methods

2.1. Cloning, expression and purification

The *aceK* gene of *E. coli* encodes isocitrate dehydrogenase kinase/phosphatase, a 68 kDa protein containing 578 amino acids. The complete *aceK* gene (gi:944797) was amplified by polymerase chain reaction using the forward primer 5′-AAA**AGATCT**AATAACAG-CGCTTTTACTTTCCAGAC-3′ and the reverse primer 5′-AA**GA-ATTC**TTAATACATAGGTGTTAATTGCAAAGG-3′. Restriction-enzyme sites for *Bgl*II and *Eco*RI (shown in bold in the primer sequences) were engineered in the forward and reverse primers, respectively. After digestion of the PCR product by *Bgl*II and *Eco*RI, the purified fragment was cloned into the *Bgl*II and *Eco*RI sites of a modified pET15b vector (Novagen). The resulting fusion protein contained a TEV protease-cleavable N-terminal extension (MGSSH-HHHHHHHHENLYFQGS) and was expressed in *E. coli* BL21 (DE3) cells. Selenomethionine AceK was expressed in the *E. coli* methionine autotroph DL41. AceK was purified to near-homogeneity using a two-step protocol. The hexahistidine tag permitted a quick and efficient first step of purification; this was followed by size-exclusion chromatography to produce a nearly homogenous protein preparation as evaluated by SDS–PAGE.

The frozen cells were suspended in 50 ml lysis buffer containing 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 0.1% Triton X-100. Cells were lysed on ice by sonication. Cell debris was removed by centrifugation for 30 min at 15 000 rev min^{-1} using a JA-25.50 rotor in a Beckman high-speed centrifuge. The clarified lysate was applied onto Ni^{2+}–nitrilotriacetic acid–agarose affinity resin (Qiagen) equilibrated with buffer *A* (30 m*M* Tris–HCl pH 8.0 and 300 m*M* NaCl) followed by a ten-column-volume wash in lysis buffer containing 20 m*M* imidazole. The protein was eluted in several fractions using a step gradient of increasing imidazole concentrations up to 300 m*M*. The fractions were analyzed using 12.5% SDS–PAGE (Laemmli, 1970). The fractions containing AceK protein were concentrated to 10 mg ml^{-1} using a Centricon-3 (Millipore) and were further purified using an ÄKTA FPLC system (Amersham Biosciences) with a size-exclusion HiLoad Superdex G-200 16/60 column in buffer containing 20 m*M* HEPES pH 7.0, 2 m*M* DTT, 100 m*M* NaCl and 10% glycerol. The major protein peak was recovered and was concentrated to 10 mg ml^{-1} using a Centricon-3 (Millipore). The purity of the concentrated protein was determined by SDS–PAGE (Fig. 1). The final yield of the protein preparation was 15 mg per litre of culture.

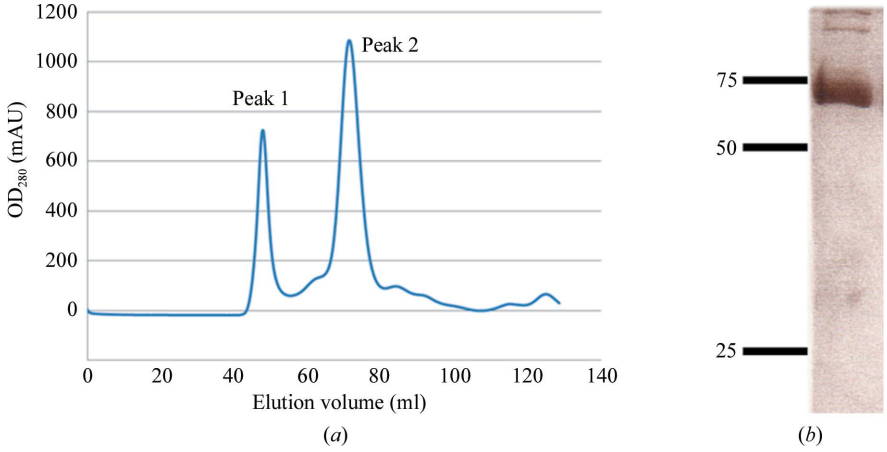

Figure 1
(*a*) AceK protein was purified using the ÄKTA FPLC system (Amersham Biosciences) with a size-exclusion HiLoad Superdex G-200 16/60 column in buffer containing 20 m*M* HEPES pH 7.0, 2 m*M* DTT, 100 m*M* NaCl and 10% glycerol. Peak 2 was pooled and concentrated to 5 mg ml^{-1} for crystallization trials. (*b*) SDS–PAGE gel analysis of purified AceK protein after size-exclusion chromatography.

Figure 2
Three forms of AceK crystals. (*a*) Tetragonal crystal, space group $P4_12_12$; the dimensions of the crystal are approximately 0.5 × 0.1 × 0.1 mm. (*b*) Hexagonal crystal, space group $P3_221$; the dimensions of the crystal are approximately 0.3 × 0.2 × 0.1 mm. (*c*) Orthorhombic crystal, space group $P2_12_12_1$; the dimensions of the crystal are approximately 0.5 × 0.1 × 0.02 mm.

Table 1
Statistics of X-ray diffraction data.

Values in parentheses are for the highest resolution shell. Two data sets for crystal form *A* were collected on the A1 and F1 beamlines at CHESS with wavelengths of 0.9789 and 0.9179 Å, respectively. One data set for crystal form *B* was collected on the A1 beamline and one data set for crystal form *C* was collected on the F1 beamline.

Crystal form	Form *A*		Form *B*	Form *C*
Wavelength (Å)	0.9789	0.9179	0.9789	0.9179
Space group	$P4_12_12$		$P3_221$	$P2_12_12_1$
Unit-cell parameters (Å)	$a = b = 124.61$, $c = 267.49$		$a = b = 152.71$, $c = 45.19$	$a = 64.20$, $b = 134.18$, $c = 187.23$
Total No. of reflections	234903	170157	67225	110674
No. of unique reflections	38959 (3829)	40192 (4059)	21542 (2093)	38426 (3730)
Resolution (Å)	30–3.1	20–3.0	50–3.0	30–2.8
Completeness (%)	99.7 (99.9)	93.7 (96.2)	93.7 (90.9)	95.1 (88.2)
$\langle I/\sigma(I)\rangle$	15.1 (3.1)	22.8 (2.9)	27.2 (2.6)	15.3 (2.4)
Redundancy	6.0 (6.2)	4.2 (4.3)	3.1 (2.8)	2.9 (2.8)
Molecules per ASU	2	2	1	2
$R_{\rm merge}$† (%)	9.7 (53.9)	8.8 (58.0)	7.6 (57.1)	10.5 (52.8)
Solvent content	0.68	0.68	0.49	0.59

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity of i observations of reflection hkl.

Selenomethionine AceK was expressed and purified using the same protocols as described above.

2.2. Crystallization

The preliminary crystallization conditions were screened by the sparse-matrix method (Jancarik & Kim, 1991) using standard screening kits. Both native and selenomethionine AceK protein were diluted to 5 mg ml^{-1} in the same buffer (20 m*M* HEPES pH 7.0, 2 m*M* DTT, 100 m*M* NaCl and 10% glycerol). ATP was added to a final concentration of 1 m*M*. The hanging-drop vapour-diffusion method was used. Hanging drops were set up to contain 2 µl protein solution mixed with 2 µl well solution. The reservoir volume was 0.5 ml. Crystals appeared in 6 d and grew to full size within two weeks. Three crystal forms were obtained from very similar crystal-growth conditions (Fig. 2). The basic optimal crystallization conditions in the reservoir consisted of 15% glycerol, 2 m*M* DTT, 0.2 *M* magnesium chloride, 0.1 *M* MES buffer (pH ranging from 5.5 to 7.0), with 12–15% PEG 8000 as the precipitating agent at room temperature. When the pH of the well solution was adjusted to 5.5 and with the addition of 1 m*M* $ZnCl_2$, a hexagonal crystal form was obtained. When the pH of the well solution pH was adjusted to 6.5 and included 1 m*M* $ZnCl_2$, a tetragonal crystal form was obtained. When the buffer pH was adjusted to 7.0 and contained 1 m*M* $CaCl_2$, an orthorhombic crystal form was obtained.

2.3. X-ray data collection

Diffraction data for the selenomethionine AceK crystals were collected on the A1 and F1 beamlines at the Cornell High Energy Synchrotron Source (CHESS; Cornell University, Ithaca, New York, USA) using an ADSC Quantum 210 CCD detector at a wavelength of 0.9789 Å at the A1 station and an ADSC Quantum 270 CCD detector at a wavelength of 0.9179 Å at the F1 station. Prior to data collection, crystals were soaked in crystallization buffer containing 25%(*v*/*v*) glycerol for 1 min followed by flash-cooling using liquid nitrogen. Single-wavelength anomalous dispersion data sets were collected with an oscillation angle of 1.0° for the hexagonal and tetragonal crystal forms at the A1 station over a total of 120°. Data sets for the tetragonal and orthorhombic crystals were collected at the F1 station. The synchrotron data were indexed and integrated using *HKL*-2000 (Otwinowski & Minor, 1997). The tetragonal crystal was determined to have $P4_12_12$ symmetry, with unit-cell parameters $a = b = 124.61$, $c = 267.49$ Å. A total of 49 741 reflections were collected in the resolution range 20–2.9 Å with an $R_{\rm merge}$ of 0.097 and a completeness of 94.4%. Calculation of the Matthews coefficient (Matthews, 1968) indicated that the asymmetric unit contained two molecules of AceK, corresponding to ∼68% solvent content (Table 1). The orthorhombic crystal was determined to have $P2_12_12_1$ symmetry, with unit-cell parameters $a = 64.20$, $b = 134.18$, $c = 187.23$ Å. A total of 44 356 reflections were collected in the resolution range 30–2.7 Å with an $R_{\rm merge}$ of 0.102 and a completeness of 93.2% (Table 1). In this crystal form, it was determined that the asymmetric unit contained two molecules of AceK, corresponding to ∼59% solvent content. The hexagonal crystal was determined to have $P3_221$ symmetry, with unit-cell parameters $a = b = 152.71$, $c = 45.19$ Å. A total of 67 225 reflections were collected in the resolution range 50–3.0 Å with an $R_{\rm merge}$ of 0.076 and a completeness of 93.7%. The asymmetric unit was determined to contain one molecule of AceK, corresponding to ∼49% solvent content (Table 1). Structure determination is currently in progress. The AceK structure will provide insight into the (de)phosphorylation regulation and mechanism and facilitate elucidation of the molecular basis underlying the bifunctionality of AceK.

3. Results and discussion

E. coli isocitrate dehydrogenase (IDH) was the first protein to be found to be regulated by a cyclic phosphorylation mechanism in *E. coli* cells by a bifunctional protein, *i.e.* AceK (Garnak & Reeves, 1979). Interestingly, the previously published crystal structure of IDH revealed that the phosphorylation site in IDH, Ser113, is positioned in the concave active site and is barely accessible to the kinase/phosphatase (Finer-Moore *et al.*, 1997). A conformational change was suspected to trigger the interaction between AceK and IDH, which might utilize a unique phosphorus-transfer mechanism (Finer-Moore *et al.*, 1997). Only the crystal structure of AceK can solve this puzzle. However, AceK has not been successfully crystallized in the 30 years since it was first reported. One possible reason is that the AceK protein is not very stable following purification. The protein sample aggregates and precipitates out after a week when stored at 277 K. We postulate that AceK has a flexible region that engages in intermolecular interactions that result in the observed aggregation. Such flexibility would enable AceK to be crystallized in three different space groups with very similar crystallization conditions and only small changes in pH.

Attempts have been made in the past to cocrystallize AceK with its substrate (IDH) in order to stabilize the putative flexible region and obtain the structure of AceK (Finer-Moore *et al.*, 1997). Unfortunately, these attempts failed, with only IDH being crystallized. In addition, it was also reported that AceK usually exists as a homodimer which is highly sensitive to *in vitro* oxidative conditions. It was implied that residues Cys67 and Cys108 from two AceK molecules connect to form a homodimer through disulfide bonding (Oudot *et al.*, 1999). For this reason, in our experiments, a high concentration of DTT (2–4 m*M*) was always maintained in the AceK protein buffer during protein purification and storage. We also noticed that the AceK protein sample could not be crystallized after storage at 193 K. Therefore, crystallization trials were always performed using freshly purified protein.

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