Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Purification, crystallization and preliminary X-ray analysis of bifunctional isocitrate dehydrogenase kinase/phosphatase in complex with its substrate, isocitrate dehydrogenase, from *Escherichia coli*

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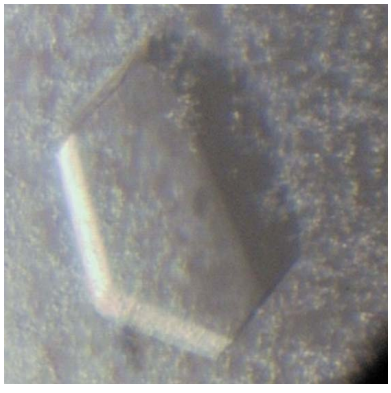
Received 23 July 2009
Accepted 24 September 2009

Escherichia coli isocitrate dehydrogenase (ICDH) can be phosphorylated and dephosphorylated by a single bifunctional protein, isocitrate dehydrogenase kinase/phosphatase (AceK), which is encoded by the *aceK* gene. In order to investigate the regulatory mechanism of (de)phosphorylation of ICDH by AceK, AceK was successfully cocrystallized in complex with its intact protein substrate, ICDH, in the presence of ATP. The complex crystal was obtained by the hanging-drop vapour-diffusion technique using PEG 300 as a precipitant and magnesium sulfate as an additive. SDS–PAGE analysis of dissolved crystals showed that the crystals contained both AceK and ICDH proteins. The complex crystals diffracted to a resolution of 2.9 Å in space group $P6_3$, with unit-cell parameters $a = b = 196.80$, $c = 156.46$ Å.

1. Introduction

In the citric acid cycle (also called the Krebs cycle), active or unphosphorylated isocitrate dehydrogenase (ICDH; EC 1.1.1.42) catalyzes the oxidative decarboxylation of isocitrate to produce α-ketoglutarate. ICDH is found to be completely inactivated by phosphorylation (LaPorte & Koshland, 1982) when nutrient conditions are deficient and acetate is the sole carbon source. With the phosphorylation of ICDH, the glyoxylate bypass is utilized to avoid the emission of two carbon dioxide molecules with the loss of energy generation during the missing steps of the citric acid cycle. In the glyoxylate bypass, isocitrate is converted to glyoxylate by isocitrate lyase and glyoxylate is further converted to malate, a downstream product of the regular citric acid cycle (Cozzone, 1998). The phosphorylation and dephosphorylation of ICDH is performed by a single protein, isocitrate dehydrogenase kinase/phosphatase or AceK (EC 2.7.11.5), which uniquely possesses both kinase and phosphatase activities (Cortay *et al.*, 1988; Klumpp *et al.*, 1988; Fig. 1). While the kinase and phosphatase activities of AceK only act on ICDH, AceK also demonstrates an ATPase activity that is independent of ICDH (LaPorte, 1993).

Phosphorylation by AceK occurs at the Ser113 residue in ICDH, which is near the active site (Hurley, Dean, Sohl *et al.*, 1990; Nimmo & Nimmo, 1984). The crystal structures of dephosphorylated (Hurley *et al.*, 1989) and phosphorylated forms of ICDH have been determined and showed that phosphorylation does not induce any significant conformational changes in the structure of ICDH (Hurley, Dean, Thorsness *et al.*, 1990). This is unlike most other regulatory phosphorylations, which may induce a conformational movement at allosteric sites (Hurley, Dean, Sohl *et al.*, 1990). The inactivation of ICDH by phosphorylation at Ser113 is a consequence of the blocking of isocitrate binding primarily by electrostatic repulsion and secondarily by steric effects (Dean & Koshland, 1990; Hurley, Dean, Sohl *et al.*, 1990). From the structures of ICDH, it is unclear how



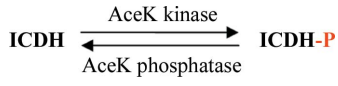


Figure 1
The reaction of AceK and ICDH in the phosphorylation/dephosphorylation process. The bifunctional enzyme AceK can phosphorylate isocitrate dehydrogenase (ICDH) to inactivate the enzyme and reversibly dephosphorylate ICDH-P to recover its activity.

AceK could access Ser113 in order to add or remove a phosphate group as this residue is buried in the core of the ICDH protein (Hurley, Dean, Sohl *et al.*, 1990). It was hypothesized that in order to bind to and transfer a phosphate to and from the Ser113 residue AceK may induce a conformational change around Ser113 or may undergo a spontaneous conformational change in ICDH that would make Ser113 accessible (Finer-Moore *et al.*, 1997).

Another interesting feature of AceK is that in contrast to many cAMP-dependent protein kinases (Knighton *et al.*, 1991), AceK cannot phosphorylate either proteolytic fragments derived from ICDH or a synthetic peptide corresponding to the sequence around the phosphorylation site. This indicates that the three-dimensional structures or the protein–protein interactions between AceK and ICDH may play an important role in enzyme–substrate recognition and specificity (McKee *et al.*, 1989). The interaction between the two proteins may be extensive and trigger a conformational change at the active site which allows the (de)phosphorylation reaction to occur. In order to reveal the structural basis of the highly specific recognition and interaction between AceK and ICDH and to investigate the mechanism of ICDH (de)phosphorylation by AceK, we have successfully crystallized the AceK–ICDH complex in the presence of ATP. Here, we describe the cloning, expression, purification, crystallization and preliminary X-ray crystallographic analysis of the AceK–ICDH complex.

2. Methods and results

2.1. Cloning, expression and purification of the AceK and ICDH proteins

Hexahistidine-tagged AceK from *Escherichia coli* was overexpressed in *E. coli* BL21 (DE3) cells and purified using Ni^{2+}–NTA affinity resin (Qiagen) followed by size-exclusion chromatography as described previously (Zheng *et al.*, 2009). The *icdH* gene (gi:1787381) in *E. coli* encodes isocitrate dehydrogenase, a 46 kDa protein containing 416 amino acids. The complete *icdH* gene was amplified by polymerase chain reaction using the forward primer 5′-AA**AGGATC**CGAAAGTAAAGTAGTTGTTCCGGCA-3′ and the reverse primer 5′-AAA**GAATTC**TTACATGTTTTCGATGATCGCGTC-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 pFO4 vector (Novagen). The resulting fusion protein contained a TEV protease-cleavable N-terminal extension (MGSSHHHHHHHHGS) and was expressed in *E. coli* BL21 (DE3) cells. The purification of ICDH was similar to the procedure used for AceK. The frozen ICDH 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+}–NTA 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 by using a step gradient of increasing imidazole concentration up to 300 m*M*. The fractions were analyzed using 12.5% SDS–PAGE. The fractions containing 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 S-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 purities of the AceK and ICDH proteins were estimated at ∼95% as shown by SDS–PAGE.

2.2. AceK–ICDH complex formation

The purified ICDH (46 kDa per subunit) and AceK (68 kDa per subunit) were mixed in a 1:1 molar ratio in the presence of 1 m*M* ATP in a buffer consisting of 100 m*M* NaCl, 20 m*M* HEPES pH 7.0, 2 m*M* DTT and 0.1% glycerol. The mixture was stored at 277 K overnight, concentrated to 5–10 mg ml^{-1} (Centricon-3, Millipore) and purified further using an ÄKTA FPLC system (Amersham Biosciences) with a size-exclusion HiLoad Superdex G-200 16/60 column in a buffer containing 25 m*M* HEPES pH 7.5, 2 m*M* DTT, 100 m*M* NaCl and 10% glycerol. The major protein peak was recovered and concentrated to 10 mg ml^{-1} (Centricon-3, Millipore). The components of the peak were determined by SDS–PAGE (Fig. 2). The final yield of the complex was ∼5 mg from 2 l *E. coli* culture.

2.3. Crystallization

The preliminary crystallization conditions for the AceK–ICDH complex were screened by the sparse-matrix method (Jancarik *et al.*, 1991) using standard screening kits. The protein was diluted to 5 mg ml^{-1} in the same protein buffer. 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 and were equilibrated against 500 µl reservoir solution at room temperature. Crystals appeared in 3 d and grew to full size within two weeks. The optimal crystallization conditions in the reservoir were 10% glycerol, 2 m*M* DTT, 0.05 *M* magnesium chloride, 0.1 *M* MES buffer pH 6.0 with 25–30% PEG 300 as the precipitating agent at room temperature. A hexagonal crystal form was obtained.

2.4. Identification of the components of the complex crystal

We harvested multiple crystals of the hexagonal crystal form in one crystallization droplet which was first diluted with 20 µl crystallization

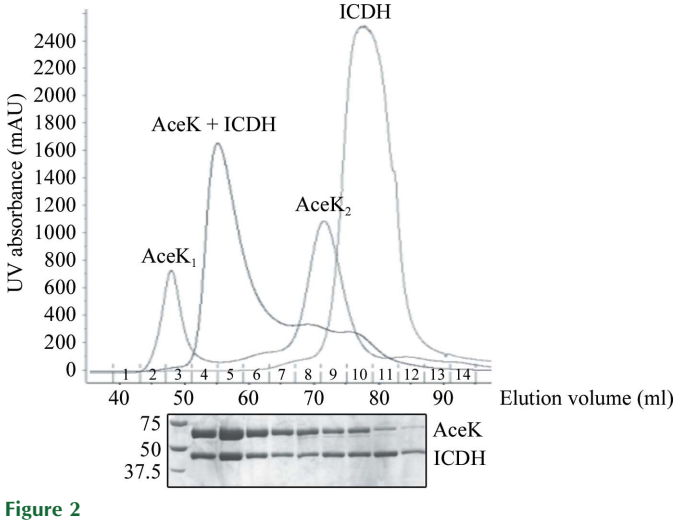


Figure 2
Binding of AceK and ICDH was demonstrated by size-exclusion chromatography. The peaks corresponding to AceK alone (two peaks), ICDH alone and the AceK–ICDH mixture are labelled. There were two AceK peaks: $AceK_1$ is aggregate and $AceK_2$ is a dimer. The eluate of the AceK–ICDH peak was analyzed by SDS–PAGE. Lanes on the gel are vertically matched to their eluting fractions. The peak fractions 4, 5 and 6 were pooled and concentrated for crystallization.

mother liquor. The complex crystals were then transferred to an Eppendorf tube using a 10 µl pipette. To remove uncrystallized protein on the surface of the crystals, the crystals were washed with crystallization mother liquor by spinning down the crystal and removing the supernatant several times. The crystals were dissolved in SDS sample buffer (2% SDS, 80 m*M* Tris pH 6.8, 10% glycerol, 0.0025% bromophenol blue) and heated at 373 K for 5 min. The components of the dissolved crystals were identified by SDS–PAGE (Fig. 3).

2.5. X-ray data collection

Diffraction data were collected from the complex crystals on the A1 beamline 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. 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. The data set was collected at 100 K with an oscillation angle of 1.0° over a total of 120°. The synchrotron data were indexed and integrated using *HKL*-2000 (Otwinowski & Minor, 1997). The hexagonal crystal was determined to be of $P6_3$ symmetry and had unit-cell parameters $a = b = 196.80$, $c = 156.46$ Å. A total of 75 640 reflections were collected in the resolution range 30–2.9 Å with an $R_{\rm merge}$ of 0.115 and a completeness of 99.6%. Calculation of the Matthews coefficient suggested that each asymmetric unit ($V_{\rm M}$ = 3.28 Å^3 Da^{-1}) contained two copies of the AceK–ICDH complex, corresponding to ∼67.1% solvent content (Table 1).

3. Discussion

As the first phosphorylation system discovered in *E. coli*, isocitrate dehydrogenase (ICDH) has several unique features. Firstly, the activity of ICDH can be controlled by a reversible phosphorylation mechanism in *E. coli* cells by a single bifunctional protein, AceK (Garnak & Reeves, 1979). However, the mechanism by which AceK is regulated to phosphorylate or dephosphorylate ICDH is still not known. Secondly, the ICDH crystal structure shows that the phosphorylation site in ICDH, Ser113, is positioned in the concave active site and is largely inaccessible to the kinase/phosphatase (Finer-Moore *et al.*, 1997), raising the question of how AceK is able to function at the IDH active site. Thirdly, the substrate specificity of AceK does not depend on the sequence motif around the phosphorylation site of ICDH, but predicatively on the overall three-demensional structure of ICDH. All these features suggest that conformational changes may occur in both AceK and ICDH which could be triggered by the interaction of AceK with ICDH or phosphorylated ICDH. Understanding these conformational changes will aid in explaining the phosphorus-transfer mechanism of AceK. Determination of the complex structure of AceK with ICDH is necessary in order to clarify the nature of the protein–protein interaction between the two proteins and reveal potential conformational changes. Attempts have been made in the past to cocrystallize AceK with its substrate ICDH (Finer-Moore *et al.*, 1997). Unfortunately, these attempts failed, with only ICDH being crystallized from the mixture. Nevertheless, it was postulated that the interface between AceK and ICDH must be extensive since AceK cannot phosphorylate proteolytic fragments derived from ICDH or synthetic peptides corresponding to the sequence around the phosphorylation site (McKee *et al.*, 1989). In most cases, enzymes and substrates have weak interactions in order to allow turnover. However, owing to the unique feature of AceK as a bifunctional protein, both ICDH and phosphorylated ICDH are the substrate and product of AceK. We thus predict that AceK may have a stronger association with ICDH. To test this, we mixed AceK and ICDH with 1 m*M* ATP but without Mg^{2+}, an important divalent ion for the phosphorylation reaction, in order to prevent the reaction. After overnight incubation at 277 K, the mixture was purified by size-exclusion chromatography. The chromatogram clearly showed a peak shift to a higher molecular weight when compared with the AceK and ICDH peaks. Furthermore, the SDS–PAGE gel from the peak showed that AceK and ICDH were both present. Interestingly, we found that the protein complex is more stable compared with AceK alone, which gradually precipitates when stored at 277 K. We predict that some flexible regions in AceK are stabilized on formation of the complex. For this reason, it was observed that the AceK–ICDH complex is more easily crystallized compared with AceK alone, despite the large total molecular weight in the asymmetric unit of the complex. The molecular weight of the AceK–ICDH complex is 114 kDa and there are two copies of the complex in the asymmetric unit, totalling 228 kDa. Structure determination of the AceK–ICDH complex is under way.

Table 1
X-ray diffraction data statistics.

Values in parentheses are for the highest resolution shell. The data set was collected on the A1 beamline at CHESS.

Wavelength (Å)	0.9789
Space group	$P6_3$
Unit-cell parameters (Å)	$a = b = 196.80$, $c = 156.46$
Total No. of reflections	75640
No. of unique reflections	38980 (3863)
Resolution (Å)	30–2.9 (3.0–2.9)
Completeness (%)	99.3 (99.4)
$\langle I/\sigma(I)\rangle$	21.8 (1.4)
Redundancy	14.9 (14.0)
Molecules per ASU	2
$R_{\rm merge}$† (%)	11.4 (57.2)
Solvent content	0.671
Matthews coefficient $V_{\rm M}$ (Å^3 Da^{-1})	3.28

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ represent the diffraction intensity values of the individual measurements and the corresponding mean values.

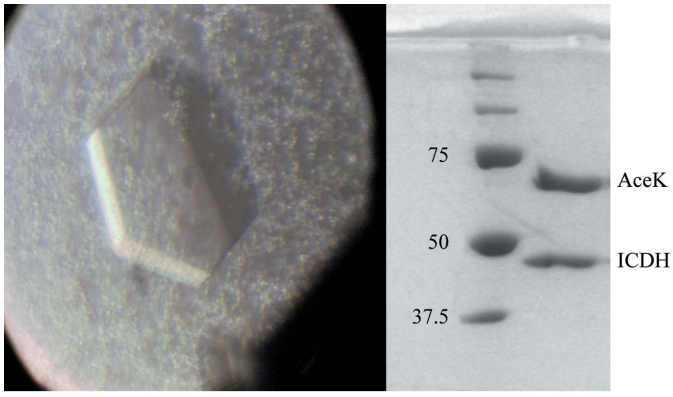


Figure 3
AceK–ICDH complex crystal. (*a*) The typical dimensions of the AceK–ICDH crystal were 0.4 × 0.3 × 0.2 mm. (*b*) SDS–PAGE analysis of dissolved AceK–ICDH crystals. The components of the complex crystal were AceK and ICDH.

The authors would like to thank Drs M. Cygler and A. Matte for their support as part of the Montreal-Kingston Bacterial Structural Genomics Initiative. This work was supported by the Canadian Institutes of Health Research and Natural Sciences and the Engi-

neering Research Council of Canada (ZJ). ZJ is a Canada Research Chair in Structural Biology.

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