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FOR THE RECORD

Crystallization of acetate kinase from *Methanosarcina thermophila* and prediction of its fold

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Abstract: The unique biochemical properties of acetate kinase present a classic conundrum in the study of the mechanism of enzyme-catalyzed phosphoryl transfer. Large, single crystals of acetate kinase from *Methanosarcina thermophila* were grown from a solution of ammonium sulfate in the presence of ATP. The crystals diffract to beyond 1.7 Å resolution. Analysis of X-ray data from the crystals is consistent with a space group of C2 and unit cell dimensions a = 181 Å, b = 67 Å, c = 83 Å, β = 103°. Diffraction data have been collected from the crystals at 110 and 277 K. Data collected at 277 K extend to lower resolution, but are more reproducible. The orientation of a noncrystallographic two-fold axis of symmetry has been determined. Based on an analysis of the predicted amino acid sequences of acetate kinase from several organisms, we hypothesize that acetate kinase is a member of the sugar kinase/actin/hsp70 structural family.

Keywords: acetate kinase; actin; acyl phosphate; covalent catalytic intermediate; glycerol kinase; hexokinase; hsp70; phosphoryl transfer

Phosphoryl transfer is the most common enzymatic function, by almost a factor of two, encoded by the yeast genome (Das et al., 1997). The catalytic cycles of enzymes such as protein kinases, G proteins, and ATPases are crucial to the ability of these molecules to regulate themselves and other proteins. Detailed understanding of the regulatory mechanisms employed by such enzymes is lacking, however, because the mechanism of phosphoryl transfer is still poorly understood (Thatcher & Kluger, 1989).

Acetate kinase is present in nearly every anaerobic microbe from both the Bacteria and Archaea domains. It has a central role in a major link in the global carbon cycle, the anaerobic decomposition of organic matter to methane (Ferry, 1997). Most of the methane is produced by microbial consortia in which fermentative anaerobes from the Bacteria domain degrade complex organic matter to acetate. Acetate kinase catalyzes the final step in this process, conversion of acetyl phosphate and ADP to acetate and ATP. Acetate can then be converted to methane and carbon dioxide by anaerobes from the Archaea domain. In this process, acetate kinase catalyzes the first step, activation of acetate to acetyl phosphate.

Although the crucial role of acetate kinase in a one-carbon metabolism is well understood (Aceti & Ferry, 1988), important questions about the mechanism of catalysis by this enzyme remain unanswered. In the presence of either ATP or acetyl-phosphate, *Escherichia coli* acetate kinase becomes phosphorylated on the side chain of one of its glutamate residues (Todhunter & Purich, 1974). The phosphoenzyme is relatively stable and can be physically isolated. The rate of phosphoenzyme formation is comparable to the rate of the overall reaction (Fox & Roseman, 1986). The isolated phosphoenzyme is able to transfer its phosphoryl group to the normal substrates, ADP and acetate (Anthony & Spector, 1970, 1972; Fox & Roseman, 1986), as well as to the active site of Enzyme I of the phosphotransferase system (Fox et al., 1986). This evidence argues that the acyl-phosphate form of the enzyme could be a covalent intermediate in catalysis. However, the available data indicate that the phosphoryl group is transferred by acetate kinase with inversion of configuration (Blättler & Knowles, 1979); this change in stereochemistry is typically taken as evidence (often the sole evidence) for a direct, in-line transfer of phosphate from substrate to product without an enzyme-linked covalent intermediate. Can these two lines of evidence be reconciled? The answer to this question is critical to a general understanding of enzymatic phosphoryl transfer, because currently the main evidence that most phosphoryl-transfer enzymes do not have covalent enzyme intermediates is stereochemical (Knowles, 1980).

There are several ways to resolve the apparent conflict between the kinetic and stereochemical observations of the acetate kinase reaction: (1) The reaction could proceed by a triple-displacement mechanism (Spector, 1980). (2) The reaction could proceed by a double-displacement mechanism, through one phosphoenzyme intermediate, in which one of the transfers does not result in inversion (Thatcher & Kluger, 1989). (3) The production of the

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phosphoenzyme could be a side reaction and not an intermediate in catalysis. Analysis of the three-dimensional structure of acetate kinase will be critical in differentiating between these possibilities.

The cloning and expression of acetate kinase from *Methanosarcina thermophila* (Aceti & Ferry, 1988; Latimer & Ferry, 1993), an acetate-utilizing methane-producing anaerobe from the Archaea domain, has inaugurated a new era in the study of acetate kinase and afforded an opportunity to reexamine mechanistic issues that were incompletely resolved in studies on acetate kinase from *E. coli*. The amino acid sequences of acetate kinase from the two organisms are 44% identical, so the two enzymes are likely to use the same catalytic mechanism. Instead of studies with a cold-labile, difficult to purify, and generally unstable enzyme, research on a thermostable and easily purified protein can now be conducted.

This study describes the growth and characterization of crystals of acetate kinase from *M. thermophila* that are well suited for X-ray analysis. Based on an analysis of the predicted amino-acid sequences of acetate kinase from several organisms, we predict that this protein is a member of the sugar kinase/actin/hsp70 family. Future determination of the structure of acetate kinase, made possible by these studies, promises to increase our understanding of the role of covalent enzyme intermediates in biological phosphoryl transfer and to provide insights into the nature of the evolution of phosphotransferases.

Results and discussion: *Data from crystals of acetate kinase at 110 and 277 K:* The acetate kinase crystals we have obtained, measuring up to 0.6 mm on a side, diffract to beyond 1.7 Å resolution. Growth of crystals is dependent on the presence of nucleotide (ATP or ADP), but is unaffected by the presence of acetate. In order to extend the lifetime of these radiation-sensitive crystals during data collection, the crystals were flash-frozen after soaking in a modified mother liquor containing 18–25% glycerol. A high-quality, high-resolution native data set collected from a frozen crystal (Table 1) will be very useful in the detailed analysis of the structure of the active site of acetate kinase.

Despite the high quality of the data from individual frozen crystals, variation among data sets from different crystals is too great to allow the collection of isomorphous derivative data useful for determination of phases by multiple isomorphous replacement. As the lack of isomorphism seems to be a result of the freezing process, unfrozen crystals may be more suitable for use in the first stages of the structure determination. A few critical changes were made in conditions of data acquisition to allow the collection of a full data set from a single acetate kinase crystal despite its radiation sensitivity. First, crystal life is extended by cooling the crystal to 4 °C with a stream of chilled air during data collection and attenuating the beam by insertion of a thicker nickel filter (0.001 mm rather than the standard 0.00015 mm). Second, data collection time is shortened by collecting wide (2°) oscillations using the lower limit of collection time for individual frames in the R-AXIS IIC system (nine minutes). Under these conditions, the typical scale factor of the last frame, after collecting 180° of data, compared to the first is 0.8. Statistics of a native data set are presented in Table 1. The R_{merge} between reflections from different native data sets is typically in the range of 5–8%.

Table 1. *Statistics collected from crystals at 110 and 277 K*

Temperature (K)	110	277
Space group	*C*2	*C*2
Unit cell dimensions		
a (Å)	179	181
b (Å)	65.8	67.4
c (Å)	82.3	82.6
β (°)	103	103
Mosaicity (°)	0.42	0.25
High resolution limit (Å)	1.70	2.0
Total observations	184,640	197,364
Unique reflections	78,834	59,879
Completeness (%)	77	91
R_{factor}[a] (%)	4.8	6.6

[a] $R_{factor} = \sum |(I - \langle I \rangle)| / \sum \langle I \rangle$.

Defining the relationship of monomers within the asymmetric unit: The content of the asymmetric unit of the acetate kinase crystals can be estimated from the unit cell dimensions of the crystals and the molecular weight of the protein calculated from its DNA sequence (44 kDa; Latimer & Ferry, 1993), assuming typical protein density in the crystals ($V_m \approx 2.5$ Å^3/Da) (Matthews, 1968). The calculated value for V_m assuming two monomers in the asymmetric unit is 2.8 Å^3/Da. Given the results of this calculation and the fact that acetate kinase from both *E. coli* and *M. thermophila* is known to form a dimer in solution (Aceti & Ferry, 1988; Fox & Roseman, 1986), it is likely that the asymmetric unit contains an intact dimer.

A self-rotation function was calculated on both native data sets described above, using the program POLARRFN (Wolfgang Kabsch) as implemented in CCP4 (Collaborative Computational Project Number 4, 1994). One solution was found (Fig. 1) that was consistent when the rotation function was calculated for both data

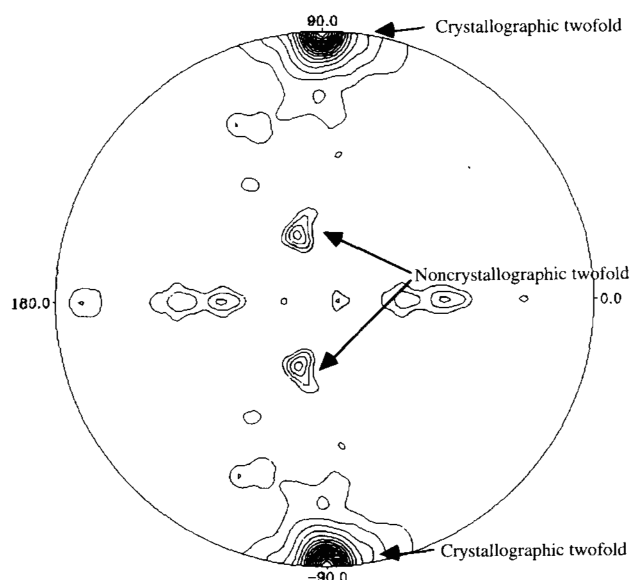

Fig. 1. Stereographic projection of the $\kappa = 180$ section of the self-rotation function. Data from 20 to 3.0 Å, collected at 277 K, was used to calculate the self-rotation function. The integration radius was 25 Å. ω (angle from pole) is 0° or 180° in the center, 90° at the edges; ϕ (angle around equator) is as marked on the periphery.

sets using various resolution ranges and integration radii and an alternate program (AMoRe; Navaza, 1994). This persistent solution indicates the presence of a twofold rotation axis at $\omega = 151°$, $\phi = 69°$, $\kappa = 180°$. This noncrystallographic twofold axis most probably relates two monomers in a dimer within the asymmetric unit.

Structure prediction from sequence data: A search of available protein sequence databases using the program BLAST (Altschul et al., 1990) found the gene encoding acetate kinase in 12 organisms, all members of the Bacteria or Archaea domains. In addition, two butyrate kinase sequences from strains of *Clostridium acetobutylicum* and a branched-chain-fatty-acid kinase from *Bacillus subtilis* were identified as homologous proteins. An unidentified open reading frame in *E. coli* (ORFX; f406) is very closely related to the acetate–kinase sequences. The sequences were aligned using the program PIMA (Smith & Smith, 1990, 1992). Conserved regions in the resulting alignment were analyzed by visual inspection, especially with regard to the question of whether this family of sequences is related to a known structural family.

A highly conserved pattern is found near the N-terminus of the acetate–kinase sequences (Fig. 2). This pattern fits well into the consensus sequence for the nucleotide-β-phosphate binding loop in the sugar kinase/hsp70/actin superfamily (Flaherty et al., 1991). The loop is between the first two β strands in the core domain of this structural superfamily, which includes hexokinase and glycerol kinase as well as hsp70 and actin. All the major features of this phosphate-binding loop are found in the acetate kinase sequences: strand-forming residues immediately surrounding loop-forming residues; a conserved residue that can act as a metal ligand (Asp in the sugar kinase/hsp70/actin family, Asn in the acetate kinases); Gly followed by Ser/Thr residues to form the loop itself; and a positively charged residue to interact with the metal ligands and phosphate (Lys in the acetate kinases). Candidates for analogues to other signature sequences in the sugar kinase/hsp70/actin superfamily (Hurley, 1996) can also be found in the acetate–kinase sequences (data not shown). Although no significant overall sequence similarity between acetate–kinase and members of the sugar kinase/hsp70/actin superfamily is detectable, neither is similarity obvious in comparison of the sequences of known members of different sub-families within the superfamily (Holmes et al., 1993). The N-terminal signature sequence is the most conserved region in the sugar kinase/hsp70/actin superfamily, and is also apparently conserved at the N-termini of the known acetate kinase sequences. We predict that the crystal structure of acetate kinase will demonstrate that it is a member of the sugar kinase/hsp70/actin structural superfamily of ATP-utilizing enzymes.

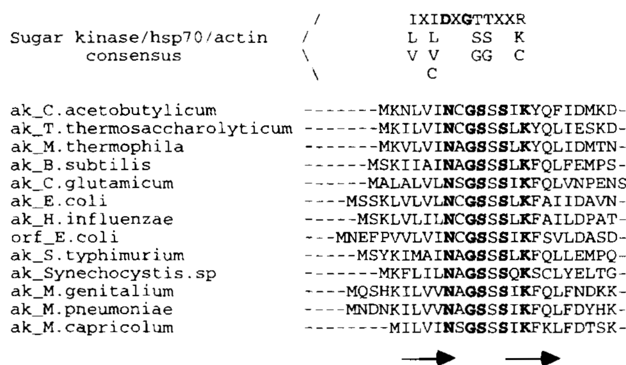

Fig. 2. Alignment of the N-terminal sequences of proteins in the acetate–kinase family, and comparison with the consensus sequence of the first phosphate-binding loop in the sugar kinase/hsp70/actin superfamily. Completely conserved residues within each family are shown in bold type. Correspondence of the two β-strands with the sequence in the sugar kinase/hsp70/actin superfamily is shown at the bottom. The sequences are arranged according to a deduced phylogenetic tree.

Materials and methods: *Crystallization:* Homogeneous acetate kinase was isolated as described previously (Aceti & Ferry, 1988; Latimer & Ferry, 1993). In a typical crystallization experiment, a 10 μL hanging drop containing 4.9 μg purified acetate kinase in a solution of 0.75 mM ATP, 0.75 mM $MgCl_2$, 315 mM $(NH_4)_2SO_4$, 0.8 mM dithiothreitol, 25 mM NaHEPES (pH 7.5) is equilibrated against a reservoir of 1.7 M $(NH_4)_2SO_4$ at room temperature.

Data collection and analysis: Data were collected using an R-AXIS IIC image plate detector and Cu-Kα X-rays generated by a Rigaku RU-200 rotating anode (50 kV × 80 mA) equipped with focusing mirrors (Molecular Structure Corporation) and a Ni filter. The set of X-ray diffraction images was reduced to integrated indexed intensities and processed with DENZO (Otwinowski & Minor, 1997) to determine the unit cell dimensions, Laue symmetry, and space group.

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References

Aceti DJ, Ferry JG. 1988. Purification and characterization of acetate kinase from acetate-grown *Methanosarcina thermophila*. *J Biol Chem 263*:15444–15448.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol 215*:403–410.

Anthony RS, Spector LB. 1970. A phosphoenzyme intermediary in acetate kinase action. *J Biol Chem 246*:6730–6741.

Anthony RS, Spector LB. 1972. Phosphorylated acetate kinase. Its isolation and reactivity. *J Biol Chem 247*:2120–2125.

Blättler WA, Knowles JR. 1979. Stereochemical course of phosphokinases. The use of adenosine [γ-(*S*)-^{16}O,^{17}O,^{18}O]triphosphate and the mechanistic consequences for the reactions catalyzed by glycerol kinase, hexokinase, pyruvate kinase, and acetate kinase. *Biochemistry 18*:3927–3933.

Collaborative Computational Project Number 4. 1994. The CCP4 suite: Programs for protein crystallography. *Acta Crystallogr D50*:760–763.

Das S, Yu LH, Gaitatzes C, Rogers R, Freeman J, Bienkowska J, Adams RM, Smith TF, Lindellen J. 1997. Biology's new rosetta stone. *Nature 385*:29–30.

Ferry JG. 1997. Enzymology of the fermentation of acetate to methane by *Methanosarcina thermophila*. *BioFactors 6*:25–35.

Flaherty KM, McKay DB, Kabsch W, Holmes KC. 1991. Similarity of the three-dimensional structures of actin and the ATPase fragment of a 70-kDa heat shock cognate protein. *Proc Natl Acad Sci USA 88*:5041–5045.

Fox DK, Meadow ND, Roseman S. 1986. Phosphate transfer between acetate kinase and Enzyme I of the bacterial phosphotransferase system. *J Biol Chem 261*:13498–13503.

Fox DK, Roseman S. 1986. Isolation and characterization of homogeneous acetate kinase from *Salmonella typhimurium* and *Escherichia coli*. *J Biol Chem 261*:13487–13497.

Holmes KC, Ander C, Valencia A. 1993. A new ATP-binding fold in actin, hexokinase and Hsc70. *Trends Cell Biol 3*:53–59.

Hurley JH. 1996. The sugar kinase/heat shock protein 70/actin superfamily—Implications of conserved structure for mechanism. *Annu Rev Biophys Biomolec Struct 25*:137–162.

Knowles JR. 1980. Enzyme-catalyzed phosphoryl transfer reactions. *Annu Rev Biochem 49*:877–919.

Latimer MT, Ferry JG. 1993. Cloning, sequence analysis, and hyperexpression of the genes encoding phosphotransacetylase and acetate kinase from *Methanosarcina thermophila. J Bacteriol 175*:6822–6829.

Matthews BW. 1968. Solvent content of protein crystals. *J Mol Biol 33*:491–497.

Navaza J. 1994. AMoRE: An automated package for molecular replacement. *Acta Crystallogr A50*:157–163.

Otwinowski Z, Minor W. 1997. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol 276*:307–326.

Smith RF, Smith TF. 1990. Automatic generation of primary sequence patterns from sets of related protein sequences. *Proc Natl Acad Sci USA 87*:118–122.

Smith RF, Smith TF. 1992. Pattern-induced multi-sequence alignment (PIMA) algorithm employing secondary structure-dependent gap penalties for use in comparative protein modelling. *Protein Eng 5*:35–41.

Spector LB. 1980. Acetate kinase: A triple-displacement enzyme. *Proc Natl Acad Sci USA 77*:2626–2630.

Thatcher GRJ, Kluger R. 1989. Mechanism and catalysis of nucleophilic substitution in phosphate esters. *Adv Phys Organic Chem 25*:99–265.

Todhunter JA, Purich DL. 1974. Evidence for the formation of a γ-phosphorylated glutamyl residue in the *Escherichia coli* acetate kinase reaction. *Biochem Biophys Res Commun 60*:273–280.