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Crystallization and preliminary X-ray analysis of pyruvate kinase type I from *Escherichia coli*.

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Abstract

Crystals of the fructose-1,6-bisphosphate-dependent pyruvate kinase from *Escherichia coli* have been grown using the hanging-drop vapour-diffusion technique. The space group was found to be $C222_1$ with cell dimensions $a = 76.8$, $b = 247.5$, $c = 132.6$ Å. Diffraction data to 3.0 Å resolution have been recorded and the enzyme molecular symmetry analysed through inspection of the self-rotation function. The crystallized protein is in the allosterically inactive T state.

Introduction

Pyruvate kinase (E.C. 2.7.1.40) catalyses the conversion of phosphoenolpyruvate (PEP) to pyruvate, coupled to the synthesis of one ATP molecule,

$$\text{PEP} + \text{ADP} + \text{H}^+ \underset{\longleftarrow}{\overset{2\text{Mg}^{2+},\text{K}^+}{\longrightarrow}} \text{ATP} + \text{pyruvate}.$$

The reaction is the last step in the glycolytic pathway and is irreversible under physiological conditions. For its activity the enzyme requires 1 equivalent of monovalent cations, normally K^+ (Boyer, Lardy & Phillips, 1942), and two equivalents of bivalent cations, usually Mg^{2+} (Gupta, Oesterling & Mildvan, 1976). Pyruvate kinase occupies a nodal point in the cellular metabolism. The product of the reaction, pyruvate, is the first non-phosphorylated intermediate in glycolysis and is involved in a number of metabolic pathways. Furthermore, the enzyme plays a major role in the regulation of glycolysis, particularly in the control of the flow from fructose-1,6-bisphosphate to pyruvate (Kayne, 1973). Not surprisingly, therefore, pyruvate kinase is a typical allosteric protein. The enzyme from a number of prokaryotes and eukaryotes has been characterised (see Fothergill & Michels, 1992) and in nearly all organisms it shows allosteric properties in binding the substrate PEP. Furthermore, the enzyme activity is frequently heterotropically regulated by one or more allosteric effectors, whose nature depends on the type of organism or tissue. The allosteric regulation is accomplished through the oligomeric organisation of the enzyme which is usually a tetramer of four identical subunits with an approximate molecular weight of 4 × 50 kDa (Fothergill & Michels, 1992).

Among bacteria, *E. coli* is peculiar for the presence of two forms of pyruvate kinase which differ in their allosteric properties. The type I isoenzyme is activated homotropically by PEP and heterotropically by fructose-1,6-bisphosphate. Moreover, it is allosterically inhibited by ATP (Markus, Plesser, Boiteux, Hess & Malcovati, 1980). The type II isoenzyme is also homotropically activated by the substrate PEP but is heterotropically activated by AMP and sugars bearing only one phosphate group (*i.e.* ribose-phosphate). Most bacteria usually have only one pyruvate kinase kinetically similar to either the *E. coli* type I or type II isoenzymes (Fothergill & Michels, 1992).

In eukaryotes, pyruvate kinase normally exhibits cooperative binding of PEP and allosteric activation by fructose-1,6-bisphosphate, therefore sharing similar properties to the *E. coli* type I isoenzyme. An exception to this pattern is given by the mammalian muscle isoenzyme M1 which shows hyperbolic Michaelis–Menten kinetics and no cooperative properties (Muirhead *et al.*, 1986). On the other hand the liver L isoenzyme is regulated not only by the allosteric effectors but also by phosphorylation of a serine residue at the N-terminus of the protein (Kayne, 1973).

The crystal structures of the non-allosteric M1 pyruvate kinase from cat (Muirhead *et al.*, 1986) and rabbit (Larsen, Laughlin, Holden, Rayment & Reed, 1994) muscles have been determined at 2.6 and 2.9 Å resolution, respectively. They show that each of the four intimately associated subunits of the tetramer is folded into three major domains, called *A*, *B* and *C*. The *A* domain is a typical $(\alpha\beta)_8$ barrel, whereas the *B* domain is mostly antiparallel β-sheet. The *C* domain comprises five α-helices and five strands of mixed β-sheet. The catalytic centre is located in a cleft defined by the *A* and *B* domains where Mg^{2+} and K^+ ions are bound.

Since the M1 pyruvate kinase has hyperbolic kinetics, the structures of this isoenzyme do not provide specific information on the allosteric properties the protein. With the aim of shedding more light into the structural bases for the mechanism of the allosteric transition and cooperative substrate binding in pyruvate kinase we have undertaken the X-ray analysis of the type I isoenzyme from *E. coli*. This protein is well characterised both in its kinetic and regulatory properties (Markus *et al.*, 1980; Valentini, Speranza, Iadarola, Ferri & Malcovati, 1988; Speranza *et al.*, 1989). The enzyme is a tetramer of four identical subunits, each consisting of 462 residues with a sequence identity of 49% with respect to the cat M1 muscle isoenzyme (Ohara, Dorit & Gilbert, 1989). A detailed analysis of the enzymatic reaction has shown that the allosteric control by ATP and fructose-1,6-bisphosphate can be described according to the sequential model of Monod, Wyman & Changeux (1965). In this paper we report the successful crystallization of the protein in the allosterically inactive T state.

Experimental procedures

Type-I pyruvate kinase was isolated from *E. coli* cells and purified as described (Valentini & Malcovati, 1982). Crystals

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of the unligated protein were grown by the hanging-drop vapour-diffusion method. The drops were formed by mixing 5 µl of the protein and 5 µl of the reservoir, where the protein solution consisted of 10 mg enzyme ml^{-1}, in 10 m*M* Tris–HCl (pH 7.5) with 2 m*M* β-mercaptoethanol and the reservoir solution contained 100 m*M* MES/NaOH (pH 6.2), 10 m*M* $MgSO_4$, 10 m*M* KCl, 0.02%(*w*/*v*) NaN_3 and 16%(*w*/*v*) PEG 8000. The presence of the cations, though not necessary for the crystallization, greatly improved the quality of the crystals. The crystallization experiments were carried out at room temperature.

For data collection at room temperature the crystals were mounted in glass capillaries with a stabilizing solution containing 25%(*w*/*v*) PEG 8000, in the same buffer as the reservoir used for crystallization. For the low-temperature data-collection experiments, the crystals were washed for a few minutes in 25%(*w*/*v*) PEG 400, 20%(*w*/*v*) PEG 8000, 100 m*M* MES–NaOH pH 6.2 and then mounted on a thread loop to be shock frozen at 100 K under a stream of nitrogen. Diffraction data were collected on a Rigaku RU-200 X-ray generator using the R-AXIS II imaging-plate system as detector. The intensities were evaluated using the program *MOSFLM* (A. G. W. Leslie) and internally scaled using the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

Results

The crystals of the unligated type I pyruvate kinase reproducibly grow in a few days, reaching a maximum size of 0.2 × 0.2 × 0.5 mm. The examination of the symmetry and systematic absences on precession photographs is consistent with the orthorhombic space group $C222_1$ with cell dimensions $a = 76.8$, $b = 247.5$, $c = 132.6$ Å. To further characterize the crystals, a native data set was collected at room temperature. A total of 68 430 intensities were evaluated and merged into a unique set of 20 988 independent reflections with an R_{merge} value of 7.3%, and a completeness of 98% up to 3.2 Å resolution. The crystals are not very stable under the X-ray beam and, therefore, cryocooling experiments were carried out in order to prevent radiation damage. A higher resolution data set could be collected at 100 K using PEG 400 as cryoprotectant. As often observed, the unit-cell dimensions shrunk by about 2% to $a = 73.9$, $b = 242.5$, $c = 129.7$ Å. The 148 480 intensities measured at low temperature were merged into a set of 26 152 independent reflections with an R_{merge} value of 8.3% and completeness of 99% up to 3.0 Å resolution. In the outer resolution shell (3.1–3.0 Å) the R_{merge} is 21% and 79% of the measured intensities are greater than $3\sigma(I)$. A test experiment at the DESY synchrotron facility (Hamburg, Germany) has indicated that the diffraction power of the crystals extends at least to 2.6 Å resolution.

A self rotation function was calculated by means of the program *GLRF* (Tong & Rossmann, 1990) using the low-temperature data in the 8.0–4.0 Å resolution range. A peak 4.5σ above the mean was obtained (Fig. 1) at $\kappa = 180$, $\varphi = 55$, $\psi = 90°$ (Rossmann & Blow, 1962), suggesting that the asymmetric unit contains a dimer of subunits. Particularly, the non-crystallographic twofold axis appears to be perpendicular to the *y* axis (Fig. 1) indicating that the tetramer has 222 symmetry with one of the molecular axes being coincident with the crystallographic *b* axis. This interpretation of the self rotation function is further confirmed by the fact that assuming half a tetramer (100 600 Da) in the asymmetric unit, the V_m value is 3.1 $Å^3\ Da^{-1}$, implying a solvent content of 60%, which is in the range of values normally found in protein crystals (Matthews, 1968).

The fact that the type I pyruvate kinase is an allosteric enzyme raises the question of which allosteric state of the protein has been crystallized. Two lines of evidence suggest that the enzyme is in the allosterically inactive T state. Firstly, all the kinetic data indicate that the unligated protein is in the inactive form (Markus *et al.*, 1980); secondly, soaking the allosteric activators, phosphenolpyruvate and fructose-1,6-bisphosphate, which induce the T-state to R-state transition, leads to the immediate cracking of the crystals. Attempts to solve the structure of type I pyruvate kinase from *E. coli* by molecular replacement based on the M1 enzyme models have so far been unsuccessful. We are now carrying out the structure determination by multiple isomorphous replacement techniques.

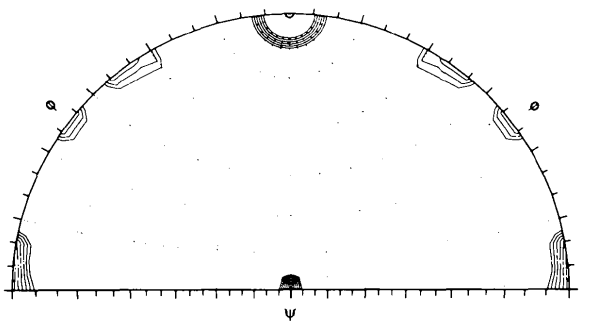

Fig. 1. Section κ = 180 of the self rotation function contoured at intervals of 1.0σ starting from 2.5σ. A total of 2663 reflections between 8.0 and 4.0 Å resolution were used in the calculation. In this projection ψ varies radially and φ varies circumferentially, whereas the crystallographic *b* axis is perpendicular to the plane of the paper and the *a* and *c* axes are horizontal and vertical, respectively. The map shows a 4.5σ peak at φ = 55, ψ = 90° and at its crystallographically related positions.

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