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Crystallization and preliminary X-ray crystallographic analysis of shikimate kinase from *Erwinia chrysanthemi*

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Abstract

Shikimate kinase from *Erwinia chrysanthemi*, overexpressed in *Escherichia coli* has been crystallized by the vapour-diffusion method using sodium chloride as a precipitant. Mass spectrometry was used to confirm the purity of the shikimate kinase and dynamic light scattering was used to assess conditions for the monodispersity of the enzyme. The crystals are tetragonal, space group $P4_12_12$ or enantiomorph with cell dimensions $a = b = 108.5$ and $c = 92.8$ Å (at 100 K). Native crystals diffract to better than 2.6 Å on a synchrotron X-ray source. The asymmetric unit is likely to contain two molecules, corresponding to a packing density of 3.6 Å^3 Da^{-1}.

1. Introduction

The shikimate pathway is essential to plants and microorganisms for the biosynthesis of aromatic compounds, but is absent from animals. The enzymes of this pathway are, therefore, targets for the development of potentially nontoxic antimicrobial agents (Davies *et al.*, 1994) and herbicides (Coggins, 1989).

Shikimate kinase (SK, E.C. 2.7.1.71) catalyses the fifth reaction in the pathway converting shikimate into its phosphorylated derivative, shikimate 3-phosphate, using ATP as a co-substrate. This reaction is catalysed by two different isoforms, a type I enzyme (SKI) and a type II enzyme (SKII). It is unusual for there to be two isoenzymes in the middle of a metabolic pathway, therefore it has been proposed that shikimate may be a branch-point intermediate for two different pathways (Weiss & Edwards, 1980). The gene for SKII has been cloned and overexpressed from *E. coli* and the enzyme was found to be a monomer (De Feyter & Pittard, 1986*a*; Millar, Lewendon, Hunter & Coggins, 1986). Recently, the gene encoding SKI from *E. coli* has also been identified (Lobner-Olsen & Marinus, 1992; Whipp & Pittard, 1995). SKII appears to play a dominant role in the shikimate pathway, (De Feyter, Davidson & Pittard, 1986) whilst the role of SKI is much less clear, as the enzyme has a much lower affinity for shikimate (K_M of 20 m*M* compared with 200 μ*M* for SKII) (De Feyter & Pittard, 1986*b*). It has been suggested that SKI may have another function in the cell and that it phosphorylates shikimate only fortuitously (De Feyter & Pittard, 1986*b*). This is consistent with the recent observation that SKI is associated with sensitivity to the antibiotic mecillinam which clearly implies an alternative biological role for SKI (Vinella, Gagny, Joseleau-Petit, D'Ari & Cashel, 1996).

Both SKI and SKII contain the type A Walker motif (Walker, Saraste, Runswick & Gay, 1982) which is shared by many ATP- and GTP-binding proteins. It has been speculated by Milner-White, Coggins & Anton (1991) that this motif could be extended to a common core structure for ATP/GTP binding proteins. The SK of *E. chrysanthemi* which was cloned and sequenced by Minton, Whitehead, Atkinson & Gilbert (1989) is a type II enzyme sharing 53% amino-acid sequence identity with the *E. coli* SKII. Matsuo & Nishikawa (1994) have predicted that *E. coli* SKII has the same fold as porcine adenylate kinase, despite the low degree of sequence similarity (19% identity).

2. Methods and results

2.1. *Enzyme purification and mass spectrometric analysis*

Shikimate kinase from *E. chrysanthemi* was overexpressed in *E. coli* BL21(DE3)pLysS to an amount of 30–40% of the total cellular protein using a T7 RNA polymerase expression system (Studier & Moffatt, 1986).

The cells (14 g) suspended in 20 ml 50 m*M* Tris–HCl, pH 7.5 containing 20 m*M* KCl, 5 m*M* $MgCl_2$ and 0.4 m*M* dithiothreitol (DTT) (buffer *A*) were broken by two passes through a French pressure cell operated at 6.9 MPa. This material was then diluted with 80 ml buffer *A* and centrifuged at 100 000*g* for 1 h. SK was purified from the resulting cell-free extract. All subsequent steps were performed at 277 K.

The supernatant was applied to a DEAE–Sephacel anion-exchange column (25 × 2.1 cm diameter, flow rate of 20 ml h^{-1}) equilibrated in buffer *A*. The column was then washed with the same buffer until the $A_{280\,nm}$ of the eluate was less than 0.3. SK was eluted with a linear gradient of 0–300 m*M* KCl in 800 ml of buffer *A* (flow rate 40 ml h^{-1}) and fractions (14 ml) collected and assayed as described in Millar *et al.* (1986).

Pooled fractions were made up to 1.2 *M* $(NH_4)_2SO_4$ by addition of solid $(NH_4)_2SO_4$ and applied to a phenyl-Sepharose hydrophobic interaction column (10 × 1.1 cm diameter, flow rate of 10 ml h^{-1}) equilibrated in 100 m*M* Tris–HCl, pH 7.5, 0.4 m*M* DTT (buffer *B*) containing 1.2 *M* $(NH_4)_2SO_4$. The column was then washed with 50 ml of buffer *B* containing 1.2 *M* $(NH_4)_2SO_4$, and SK was eluted with a linear gradient (200 ml) of 1.2–0.0 *M* $(NH_4)_2SO_4$ in buffer *B*. The flow rate was 10 ml h^{-1} and 4 ml fractions were collected and assayed. Fractions containing SK activity were pooled and concentrated by vacuum dialysis. At this stage a substantial amount of enzyme was lost due to its irreversible precipitation.

The concentrated enzyme solution was dialysed against 50 m*M* Tris–HCl, pH 7.5 containing 500 m*M* KCl, 0.4 m*M* DTT and 10%(*v*/*v*) glycerol (buffer *C*) and applied to a Sephacryl S200 (superfine grade) column (85 × 2.5 cm diameter, flow rate of 8 ml h^{-1}) that had been equilibrated with buffer *C*. The enzyme was eluted with buffer *C* (flow rate 8 ml h^{-1}, 4 ml fractions). Fractions containing SK activity were dialysed against buffer *A* containing 50%(*v*/*v*) glycerol before long-term storage at 253 K.

Mass spectrometry was performed on a VG Platform quadrupole mass spectrometer fitted with a pneumatically assisted electrospray source (VG Biotech Ltd, Altrincham, Cheshire, UK). For experimental details see Krell, Pitt & Coggins (1995).

The protein to be crystallized was shown to be of high purity using electrospray mass spectrometry (Fig. 1). Two different enzyme species were detected which corresponded to full-length enzyme (60%) and another species where the N-terminal methionine had been cleaved off (40%). This heterogeneity can be explained by a lack of complete processing of the enzyme by the cell due to the very high level of overexpression.

2.2. *Dynamic light scattering*

Experiments were carried out using a DYNA-PRO 801 dynamic light-scattering/molecular-sizing instrument (Protein Solutions, Buckinghamshire, UK). Protein solutions (1 mg ml^{-1}) were in 50 m*M* Tris–HCl, pH 7.6. Dynamic light-scattering experiments were carried out in parallel with crystallization trials to assess conditions for the monodispersity of the enzyme. Several conditions were tried (Table 1), however, it was shown that monodispersity could only be achieved in the presence of the substrate shikimate, ADP and $MgCl_2$. Under those conditions the estimated molecular weight obtained, 18 kDa, was consistent with a monomeric state for the enzyme in solution. Furthermore, the solubility of the enzyme was found to be greatly increased in the presence of shikimate, ADP and $MgCl_2$ (from 5 mg ml^{-1} in buffer to at least 70 mg ml^{-1}). In the light of these results crystallization trials were carried out in the presence of these compounds.

2.3. *Crystallization*

A wide variety of crystallization conditions were tested using sparse-matrix screens (Jancarik & Kim, 1991). Crystallizations were performed at room temperature using the sitting-drop vapour-diffusion technique.

The enzyme was dialysed exhaustively into 20 m*M* Tris–HCl, pH 7.6. Shikimate and ADP (freshly made up solution in water, pH adjusted to 7.6) were added at a final concentration of 5 m*M* and $MgCl_2$ to a final concentration of 10 m*M*. Enzyme was concentrated using Centricon-10 centrifugal concentrators (Amicon, Stonehouse, Gloucestershire, UK) to a concentration of 16 mg ml^{-1}. Initial crystals were obtained from condition 36 of the Hampton Research crystal screen II (Cudney, Patel, Weisgraber, Newhouse & McPherson, 1994). These crystallization conditions were optimized to 2.16 *M* sodium chloride, 100 m*M* Hepes buffer, pH 6.9. Typically, 6 µl of protein (16 mg ml^{-1}) were mixed with an equal volume of the reservoir solution. Crystals appear after 10–12 d and continue to grow as tetragonal bipyramids up to a maximum size of 0.7 × 0.2 × 0.2 mm (Fig. 2).

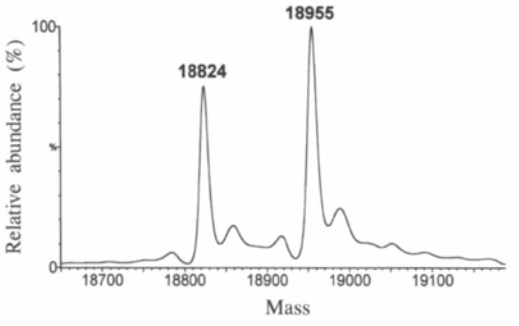

Fig. 1. MaxEnt deconvolution mass spectra of recombinant shikimate kinase from *Erwinia chrysanthemi*. MaxEnt spectra can be used for a semi-quantitative analysis by comparing integrated peak areas (Ferrige, Seddon, Green, Jarvis & Skilling, 1992). The crystallized protein was heterogeneous: the peak with the molecular weight (M_r) of 18 955 confirms the theoretical M_r of 18 955.85 as deduced from the nucleotide sequence (Minton *et al.*, 1989). The peak with M_r of 18 824 corresponds to enzyme species with the N-terminal methionine residue cleaved off (mass difference of 131). The small peaks to the right of the major peaks are potassium adducts (mass difference 39).

2.4. *X-ray analysis*

The X-ray diffraction data were collected on beamline 7.2 at the SRS Daresbury Laboratory using a wavelength of 1.44 Å and a MAR Research imaging-plate system. The crystals were radiation sensitive, therefore cryo-cooling was essential.

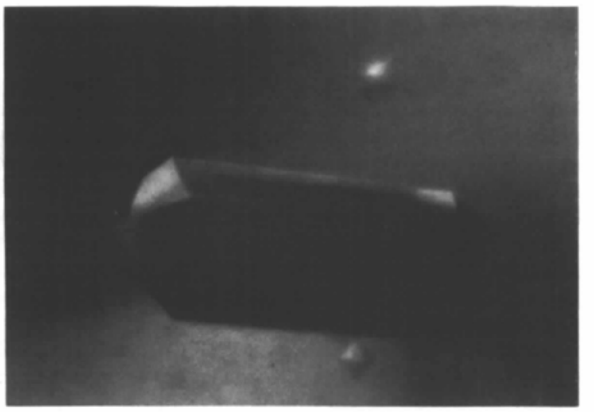

Fig. 2. A crystal of shikimate kinase from *Erwinia chrysanthemi*. The size is *ca* 0.6 × 0.175 × 0.175 mm.

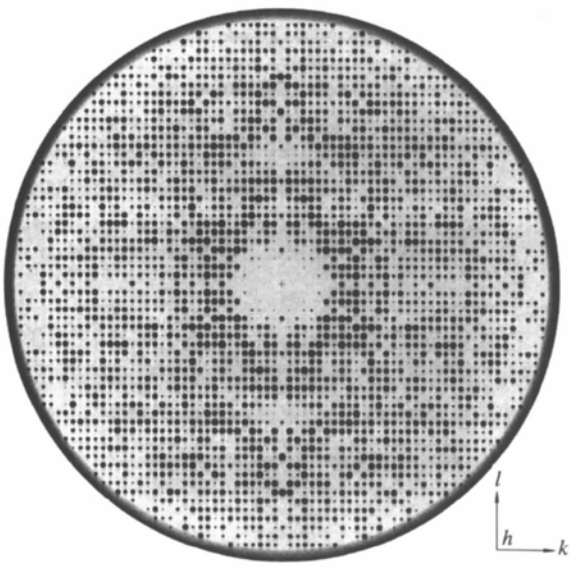

Fig. 3. A pseudo precession plot of the *h0l* zone extending to 3.0 Å calculated using the *PATTERN* program (G. Lu, unpublished work) showing the systematic absences.

Table 1. *Dynamic light-scattering measurements of E. chrysanthemi shikimate kinase in the presence of different additives*

The hydrodynamic radius represents the median particle size present in the sample cell. The polydispersity value indicates the standard deviation of the spread of particle sizes about the reported average radius. A rise in polydispersity in relation to the average radius represents greater spread in the size distribution. The estimated M_r is calculated from the hydrodynamic radius (R_H) using an empirically derived relationship between the R_H and M_r values for a number of well characterized globular proteins in buffered solution.

Additives	Hydrodynamic radius (nm)	Polydispersity (nm)	Estimated M_r (kDa)	Conclusion
Shikimate (5 m*M*) ADP (5 m*M*) $MgCl_2$ (10 m*M*)	21. (0)	0.477 (02)	18	Monodisperse
ADP (5 m*M*) $MgCl_2$ (10 m*M*)	3.0 (0)	1.698 (01)	43	Polydisperse
Shikimate (5 m*M*)	2.7 (1)	1.193 (2)	30	Polydisperse

Crystals were loopmounted in a cryoprotectant containing 17.5%(*v*/*v*) glycerol and cryo-cooled to 100 K using an Oxford Cryosystems Cryostream. A native data set to 2.6 Å resolution was collected using 1° oscillation frames. Data were processed with *DENZO* and scaled with *SCALEPACK* (Otwinowski, 1993). From autoindexing by *DENZO* the crystals were found to belong to the tetragonal crystal system, with unit-cell dimensions of $a = b = 108.5$ and $c = 92.8$ Å. Analysis of the systematic absences in the data revealed absences at $h = 2$ and $l = 4n$ along the (h00) and (00l) axes, respectively, which are consistent with the space group of $P4_12_12$ or enantiomorph (Fig. 3). An assumption of two molecules per asymmetric unit leads to an acceptable packing density, V_m of 3.6 $Å^3\ Da^{-1}$, corresponding to a solvent content of 66% (Matthews, 1968). Interestingly, the self-rotation function gives no indication of a non-crystallographic twofold axis. Repeated attempts to solve the structure by molecular replacement with *AMoRe* (Navaza, 1990), using porcine adenylate kinase as a search model, were unsuccessful. We are currently using multiple isomorphous replacement methods to solve the structure.

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References

Coggins, J. R. (1989). In *Herbicides and Plant Metabolism*, edited by A. Dodge. Cambridge University Press.

Cudney, R., Patel, S., Weisgraber, K., Newhouse, Y. & McPherson, A. (1994). *Acta Cryst.* D**50**, 414–423.

Davies, G. M., Barrett-Bee, K. J., Jude, D. A., Lehan, M., Nichols, W. W., Pinder, P. E., Thain, J. L., Watkins, W. J. & Wilson, R. G. (1994). *Antimicrob. Agents Chemother.* **38**, 403–406.

De Feyter, R. C., Davidson, B. E. & Pittard, J. (1986). *J. Bacteriol.* **165**, 233–239.

De Feyter, R. C. & Pittard, J. (1986*a*). *J. Bacteriol.* **165**, 226–232.

De Feyter, R. C. & Pittard, J. (1986*b*). *J. Bacteriol.* **165**, 331–333.

Ferrige, A. G., Seddon, M. J., Green, B. N., Jarvis, S. A. & Skilling, J. (1992). *Rapid Commun. Mass Spectrom.* **6**, 707–711.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 299–329.

Krell, T., Pitt, A. R. & Coggins, J. R. (1995). *FEBS Lett.* **360**, 93–96.

Lobner-Olsen, A. & Marinus, M. G. (1992). *J. Bacteriol.* **174**, 525–529.

Matsuo, Y. & Nishikawa, K. (1994). *Protein Sci.* **3**, 2055–2063.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Millar, G., Lewendon, A., Hunter, M. G. & Coggins, J. R. (1986). *Biochem. J.* **237**, 427–437.

Milner-White, J. E., Coggins, J. R. & Anton, I. A. (1991). *J. Mol. Biol.* **221**, 751–754.

Minton, N. P., Whitehead, P. J., Atkinson, T. & Gilbert, H. J. (1989). *Nucleic Acids Res.* **17**, 1769.

Navaza, J. (1990). *Acta Cryst.* A**46**, 619–620.

Otwinowski, Z. (1993). *Data Collection and Processing. Proceedings of the CCP4 Study Weekend*, pp. 56–62. Warrington, England: Daresbury Laboratory.

Studier, F. W. & Moffatt, B. A. (1986). *J. Mol. Biol.* **189**, 113–130.

Vinella, D., Gagny, B., Joseleau-Petit, D., D'Ari, R. & Cashel, M. (1996). *J. Bacteriol.* **178**, 3818–3828.

Walker, J. E., Saraste, M., Runswick, M. J. & Gay, N. J. (1982). *EMBO J.* **1**, 945–951.

Weiss, U. & Edwards, J. M. (1980). *The Biosynthesis of Aromatic Compounds*, pp. 103–133. New York: John Wiley.

Whipp, M. J. & Pittard, A. J. (1995). *J. Bacteriol.* **177**, 1627–1629.