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Isocitrate lyase from *Aspergillus nidulans*: crystallization and X-ray analysis of a glyoxylate cycle enzyme

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Abstract

Isocitrate lyase (ICL) from the filamentous fungus *Aspergillus nidulans* catalyzes the first committed step of the carbon-conserving glyoxylate bypass. This enzyme has been crystallized by the hanging-drop method of vapour diffusion using polyethylene glycol 2000 as the precipitant. Diffraction patterns show that the crystals diffract to beyond 2.5 Å and are probably in space group $P4_22_12$ with unit-cell dimensions of $a = b = 91.9$ and $c = 152.7$ Å, with one molecule in the asymmetric unit. The elucidation of the structure of this enzyme to high resolution will advance the understanding of how the metabolic branch point between the tricarboxylic acid cycle and the glyoxylate bypass is controlled by the affinity of ICL for its substrate isocitrate and contribute to a programme of rational drug design.

1. Introduction

Isocitrate lyase [threo-D_s-isocitrate-glyoxylate-lyase (ICL) E.C. 4.1.3.1] catalyzes the Mg^{2+}/Mn^{2+}-dependent reversible cleavage of isocitrate into succinate and glyoxylate,

$$\text{isocitrate} \overset{Mg^{2+}/Mn^{2+}}{\longleftrightarrow} \text{succinate} + \text{glyoxylate}.$$

ICL functions at a branch point of the tricarboxylic acid (TCA) cycle, catalysing the first unique step of the glyoxylate bypass, which diverts isocitrate through a carbon-conserving pathway. ICL and malate synthase (MS), the two enzymes involved in the glyoxylate bypass, are induced in microorganisms in response to growth on C2 compounds, such as acetate, as the sole carbon source.

The operation of this pathway diverts isocitrate from its utilization by the enzyme isocitrate dehydrogenase (ICDH) within the TCA cycle, effectively replenishing the metabolic intermediates necessary for cell growth. The crucial elements in the control of this key metabolic branch point in *Escherichia coli* are the K_m values of ICL and ICDH for isocitrate and its intracellular concentration, as well as the inactivation of ICDH through phosphorylation of an active-site serine residue, S113. This phosphorylation results in inhibition of ICDH and an increase in intracellular isocitrate which in turn allows flux through ICL which has a much higher K_m for isocitrate under normal physiological conditions than does ICDH (Nimmo *et al.*, 1987).

ICL and enzymes from the glyoxylate bypass offer an attractive target for the development of new pharmaceuticals because the bypass is inactive in human hosts but operative in *Pseudomonas aeroginosa*, a major contributor to pathogenesis in cystic fibrosis (Shimamoto & Berk, 1980), *Mycobacterium leprae*, the organism responsible for leprosy and species of *Leishmania*, an insidious human parasite (Simon, Martin & Mukkada, 1978). Furthermore, two tight binding inhibitors of ICL are already known, itaconate (McFadden & Purohit, 1977; Vanni, Giachetti, Pinzauti & McFadden, 1990) and 3-nitropropionate (Schloss & Cleland, 1982). Both these compounds are thought to mimic intermediates formed during the catalytic cycle, and therefore inhibitor binding studies could well provide important insights into the enzyme mechanism as well as contributing to a programme of rational drug design.

ICL is widely distributed amongst bacteria, fungi and algae as well as among higher plants (Vanni *et al.*, 1990). However, in contrast to the ICL from prokaryotes, which is found in the cytoplasm (McFadden & Howes 1962; Cioni, Pinzauti & Vanni, 1981), ICL in eukaryotes is located in microbodies termed glyoxysomes (Cioni *et al.*, 1981; Trelase, 1984). Biochemical studies have established that the majority of ICL's are tetrameric including the enzymes from *Aspergillus nidulans* (De Lucas, Amor, Daz, Turner & Laborda, 1997), *Pseudomonas indigofera* (McFadden, Rao, Cohen & Roche, 1968) and *E. coli* (Robertson & Reeves, 1987). However, the ICL's from *Candida tropicalis* (Uchida *et al.*, 1986) and *Glycine max* (Ruchti & Widmer, 1986) are thought to be dimeric whilst that of *Pinus densiflora* is proposed to be trimeric (Tsukamoto, Ejiri & Katsumata, 1986). The subunit molecular weights of the prokaryotic enzymes are around $M_r = 48\,000$, whilst the M_r values of plant ICL's vary between 62 000 and 67 000 and the nematode enzyme appears to have a much larger subunit of 120 000 (Vanni *et al.*, 1990). The ICL from *A. nidulans* is a homo-tetramer with a subunit M_r of 59 000 (De Lucas *et al.*, 1997).

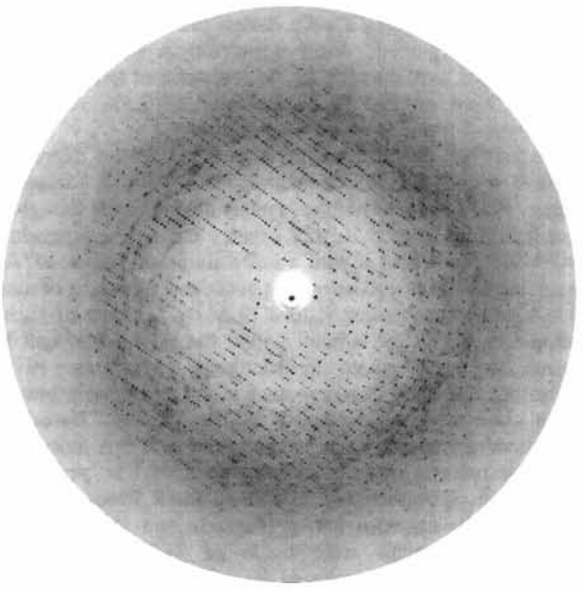
Previous attempts to crystallize ICL have been problematic; successful crystallization of the *E. coli* enzyme was only reported following chemical modification of the enzyme by either using 3-bromopyruvate or mercurial reagents such as ethyl mercuri thiosalicylate (EMTS) (Abeysinghe *et al.*, 1991). Microcrystals have been observed of *Pseudomonas indigofera* ICL (McFadden & Howes, 1963), whilst X-ray diffraction quality crystals of ICL have been reported following successful crystallization trials aboard a NASA space shuttle (DeLucas *et al.*, 1989; DeMattei, Feigelson & Weber, 1992). To date, there has been no report of any structure for ICL and, therefore, we have attempted to isolate and crystallize the enzyme from other species in order to produce crystals suitable for structural studies. This paper reports the production of crystals of *A. nidulans* ICL and their preliminary X-ray analysis.

2. Experimental

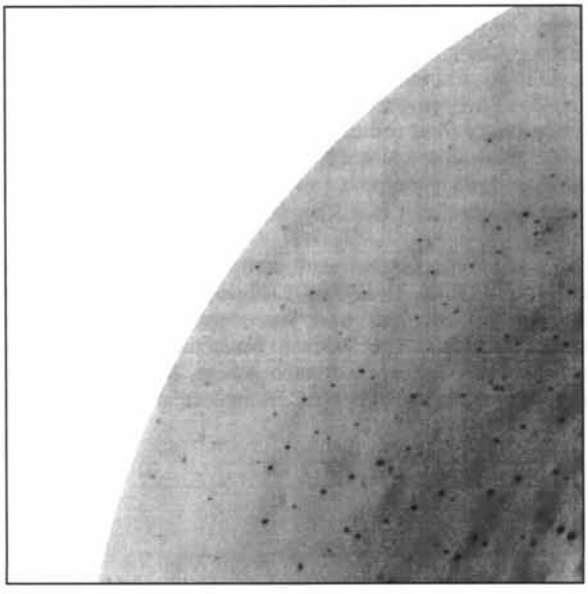
The *A. nidulans* ICL over-producing strain JCB4a was obtained by transformation of *A. nidulans* 400-4 (pyrG89, pabaA1; acuD320; wA3; uaY9) with the plasmid pDJB11 containing the structural gene for ICL plus necessary control regions (Ballance & Turner, 1986). This over-producing strain contains multiple copies of the *acuD* (ICL) gene integrated into the genome (De Lucas *et al.*, 1997). *A. nidulans* JCB4a was grown in *Aspergillus* minimal medium with acetate as the sole carbon source (Pontecorvo, Roper, Hammons, Macdonal & Bufton, 1953) as described previously (De Lucas *et al.*, 1997). Mycelia were harvested by filtration through Mira-cloth (Calbiochem-Novabiochem, USA) and washed several times with buffer *A* (0.1 *M* HEPES, 5 m*M* $MgCl_2$, 1 m*M* phenylmethyl-sulfonyl fluoride (PMSF), 2 m*M* dithiothreitol (DTT), pH 7.5), cooled on ice briefly before being flash frozen in liquid nitrogen. Frozen mycelia were ground in liquid nitrogen to a fine powder with a pestle and mortar before being resuspended in buffer *B* (0.1 *M* HEPES, 5 m*M* $MgCl_2$, 1 m*M* PMSF, 2 m*M* DTT, pH 8.5). This homogenate was filtered through muslin tissue to remove any large pieces of cell debris and the filtrate was treated with protamine sulfate (50 mg ml^{-1}) to remove any DNA contamination. This solution was clarified by centrifugation at 19 000*g* for 30 min at 277 K and the supernatant decanted and diluted twofold in buffer *C* [40 m*M* Tris–HCL, 2 m*M* ethylenediaminetetraacetic acid (EDTA), pH 8.5].

All of the following purification steps were carried out at 277 K. The crude extract was applied to a column packed with DEAE–Sepharose Fast Flow matrix (Pharmacia) previously equilibrated with buffer *C*. The column was washed with buffer *C*, after which ammonium sulfate was added to 65% saturation to the pass-through fraction (which contained the ICL) to precipitate the enzyme. The pellet was recovered by centrifugation at 19 000*g* for 25 min and resuspended in buffer *D* (40 m*M* Tris–HCL, pH 8.5). Ammonium sulfate was added up to 1.5 *M* before the sample was loaded onto a Butyl-Toyopearl 650S column (Tosoh Corporation, Japan). The ICL was eluted by a reverse gradient of ammonium sulfate concentration from 1.5 to 0 *M* in buffer *C*, with the ICL enzyme being eluted as a sharp peak at 1 *M* ammonium sulfate. Peak fractions were collected and the ICL precipitated by adding 250 mg ml^{-1} of ground ammonium sulfate powder. The pellet was recovered by centrifugation at 19 000*g* for 25 min and resuspended in 2 ml of buffer *E* (0.1 *M* HEPES, 5 m*M* $MgCl_2$, pH 7), before being loaded onto a gel-filtration column pre-packed with Superdex-200 prep grade (Pharmacia) and previously washed with buffer *E*. The gel-filtration step was run with buffer *E* using a flow rate of 0.1 ml min^{-1}, after which the peak fractions were pooled and the purity of the ICL was estimated to be greater than 95% by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

For crystallization, the purified protein concentration was adjusted to approximately 15 mg ml^{-1} using an Amicon Centricon 30 microconcentrator by centrifugation at 4000*g*. Crystallization trials were conducted by mixing 5 µl of the concentrated protein solution with an equal volume of polyethylene glycol 2000 solution in 0.1 *M* Na-HEPES at pH 7.5 in the range 1–20%(*w*/*v*) and slowly concentrated at 290 K using the hanging-drop method of vapour diffusion. Crystals were obtained after 24–36 h with polyethylene glycol 2000 as the precipitant over the concentration range of 4–8%(*w*/*v*). The crystal morphology was that of tetragonal bipyramids with maximal dimensions of 0.8 × 0.6 × 0.6 mm. The crystals were confirmed to be that of *A. nidulans* ICL by removing a crystal directly from a hanging drop and washing it briefly in fresh 10% polyethylene glycol 2000 solution in 0.1 *M* Na-HEPES at pH 7.5 and dialysing it against 5 m*M* Na-HEPES buffer at pH 7.5 over several days to remove any polyethylene glycol 2000. Direct sequencing of the dissolved protein crystal revealed that the N-terminus was blocked. However, the crystals were



(*a*)



(*b*)

Fig. 1. (*a*) A 1.5° oscillation image of a crystal of *A. nidulans* ICL taken using a MAR Research image plate on station 9.6 at CCLRC Daresbury Laboratory Synchrotron. The resolution at the edge of the image is 2.3 Å. (*b*) An enlarged area of (*a*) showing the edge of the Mar Research image plate which is at 2.3 Å resolution.

identified as ICL by sequencing two fragments isolated from a partial cyanogen bromide (CNBR) digest on an Applied Biosystems 476A sequencer.

3. Results and discussion

A preliminary native data set was collected to 3.1 Å from a single crystal on a twin San Diego multiwire systems (SDMS) area detector (Hamlin 1985; Xuong, Nielsen, Hamlin & Anderson, 1985), using Cu $K\alpha$ X-rays produced by a Rigaku RU-200 rotating-anode X-ray generator. A total of 78 205 measurements were made of 12 279 independent reflections and the data were merged to an R factor of 7.5% with 87% completeness from 20 to 3.1 Å resolution.

Analysis of the data using the autoindexing algorithm supplied with the SDMS detector system (Howard, Nielsen & Xuong, 1985) showed that the crystals belong to the tetragonal system, point group $P422$ with unit-cell dimensions $a = b = 91.9$, $c = 152.7$ Å with a cell volume of 1.29×10^6 Å^3. Assuming that the asymmetric unit contains a monomer of mass 59 kDa, the corresponding V_m is 2.73 Å^3 Da^{-1}. Values of V_m for multiple copies of the subunit within the asymmetric unit lie outside the range given by Matthews (Matthews, 1977), suggesting that there is a monomer in the asymmetric unit. A native data set was collected at the CCLRC Daresbury Laboratory Synchrotron on station 9.6 to 2.5 Å resolution using the rotation method with rotations of 1.5° per frame. Observations were recorded on a small MAR Research image plate at a wavelength of 0.87 Å (Figs. 1*a* and 1*b*). A total of 61 492 measurements were made of 21 252 independent reflections and the data merged to an R factor of 4.9% with 91.5% completeness from 20 to 2.5 Å resolution. Examination of these data revealed that the axial reflections are present only when $l = 2n$ or $h = k = 2n$ identifying the space group as $P4_22_12$. Since *A. nidulans* ICL is known to be a tetramer, considerations of the space-group symmetry would demand that the four subunits are arranged in 222 symmetry about the 222 point in the lattice, assuming there is a monomer per asymmetric unit. The relatively short cell dimensions of the crystals, their strong diffraction on conventional X-ray sources and their resistance to radiation damage permit a rapid screen for derivatives to take place 'in-house' and this is currently under way.

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