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Preliminary crystallographic studies of citrate synthase from an Antarctic psychrotolerant bacterium

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Abstract

Recombinant citrate synthase from a psychrotolerant bacterium, DS2–3R, recently isolated in Antarctica, has been crystallized. The crystals belong to space group $P6_122$ or $P6_522$, with cell dimensions $a = b = 70.8$, $c = 307.8$ Å. Diffraction data collected on a synchrotron from a cryoprotected crystal extend to at least 2.0 Å. Knowledge of the structure of this enzyme will add to the understanding of cold activity and thermolability, and will be of biotechnological interest. Previously, the structure of citrate synthase from Archaea inhabiting environments at 328 and 373 K, has been reported. This present study will extend our understanding of the structural integrity and activity of proteins at the temperature extremes of life.

1. Introduction

DS2–3R is a psychrotolerant bacterium that inhabits Antarctica, and, therefore, must posses adaptational mechanisms to withstand and flourish in permanently cold temperatures. The biotechnological potential of such adaptations has been recognised (Herbert, 1992). The enzyme citrate synthase, which catalyses the entry of carbon into the citric acid cycle, provides a good model enzyme to study structure–activity relationships of proteins from extremophiles, as there is a wealth of sequence, structural and kinetic data for this enzyme from a variety of organisms.

X-ray structures for the dimeric form of citrate synthase have been elucidated for pig/chicken (Remington *et al.*, 1982, Liao *et al.*, 1991), *Thermoplasma acidophilum* (Russell *et al.* 1994), a thermophilic Archaeon that grows optimally at 328 K, and *Pyrococcus furiosus* (Russell *et al.*, 1997), a hyperthermophilic Archaeon growing optimally at 373 K. Therefore, structures exist for citrate synthase spanning mesophilic temperatures, through thermophilic to hyperthermophilic temperatures. These studies have revealed an increase in compactness of the enzyme as you ascend the temperature scale and alterations in the subunit interface of the enzymes, with a high number of complex ion-pair networks present in the hyperthermophilic enzyme. The latter was also observed in the hyperthermophilic glutamate dehydrogenase from *Pyrococcus furiosus* (Yip *et al.*, 1995). To extend this temperature range, the citrate synthase from DS2–3R was isolated. The characteristics of this enzyme reflect its origin from a psychrotolerant host. The temperature optimum for activity is 304 K and it is thermally inactivated at low temperatures (*e.g.* at 318 K, $t_{1/2} = 8$ min). By comparison, citrate synthase from *P. furiosus* has a temperature optimum >363 K, and loses half of its activity within 8 min at 373 K. Pig citrate synthase has a temperature optimum of 328 K, reflecting the common observation that optimal enzyme activity is at higher temperatures than the optimal growth temperature.

The citrate synthases of DS2–3R and *P. furiosus* share 40% sequence identity, and a number of sequence differences have been identified which may explain their widely differing stabilities and temperature optima. Although molecular modelling and sequence analyses have revealed some possible determinants of thermolability and cold-activity, such as increased flexibility of the protein due to a reduction in the number of proline residues, loop extensions and a decreased number of ion-pair interactions (Davail *et al.*, 1994; Feller *et al.*, 1996), the insights which can be gained from such studies are limited. Crystallographic studies have, therefore, been initiated to understand the molecular mechanisms behind cold-activity and thermolability.

2. Crystallization and data collection

The citrate synthase gene was cloned from the psychrotolerant bacterium DS2–3R, expressed in *E. coli*, and the protein purified as described by Gerike *et al.* (1997). Enzyme in 20 m*M* Tris, pH 8.0, 100 m*M* KCl was incubated for 5 h on ice with 10 m*M* CoASH and 10 m*M* citrate. Initial crystallization conditions were screened using the sparse-matrix method (Jancarik & Kim, 1991) by the hanging-drop vapour-diffusion method at a protein concentration of 5 mg ml^{-1}. Long branched crystals appeared overnight at 293 K in precipitant 14 of the Hampton Research Screen II [2 *M* $(NH_4)_2SO_4$, 0.1 *M* Na citrate pH 5.6, 0.2 *M* Na^+/K^+ tartrate]. Buffers containing 30% PEG 4000 as well as 1.6 *M* citrate pH 5.6 also resulted in crystals. Optimization of the latter conditions did not give good quality crystals and hence are not further described.

The protein concentration was varied from 5 to 30 mg ml^{-1} and showed that with dilution a smaller number of thinner crystals appeared. 15 mg ml^{-1} was found to be the optimal concentration in terms of crystal size. Na^+/K^+ tartrate did not affect crystal formation and was omitted from subsequent trials. Further optimization included the variation of the concentrations of $(NH_4)_2SO_4$ (1–3 *M*) and citrate (0–400 m*M*) as well as pH (5.2–6.4). Crystals formed between 1.6 and 2.2 *M* $(NH_4)_2SO_4$ with 2.2 *M* being the optimal concentration as judged from the occurrence of single unbranched crystals. Above 2.6 *M* $(NH_4)_2SO_4$ the protein precipitated. The presence of citrate was necessary to prevent precipitation. Increasing the citrate concentration to 200 m*M* resulted in more crystals but the size and thickness did not increase, whereas at 400 m*M* citrate the protein precipitated. Protein precipitation also occurred at a pH < 5.6 and pH > 6.0.

Different temperatures were investigated showing that low temperature (277 K) just increased the time (2 months) of

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Table 1. *Data-collection statistics*

Values in parentheses refer to reflections in the outer resolution shell, 4.04–3.90 Å for the in-house data, and 2.16–2.09 Å for the Hamburg data.

	In-house	Hamburg
No. of measured reflections	47577	293261
No. of unique reflections	4035	27243
Resolution (Å)	3.9	2.09
R_{merge} (%)†	13.3 (14.8)	5.0 (12.2)
Completeness (%)	84.6 (88.3)	96.2 (88.3)
$I/\sigma(I)$	13.2 (11.4)	23.9 (7.7)

† $R_{merge} = \sum |I(k) - \langle I \rangle| / \sum I(k)$, where $I(k)$ is the value of the kth measurement of the intensity of a reflection, $\langle I \rangle$ is the mean value of the intensity of that reflection, and the summation is over all measurements.

crystal formation with no difference in the size and thickness. There was no apparent difference in the appearance of crystals grown at 293 or 302 K, but the best diffraction data were obtained from crystals grown at the higher temperature. The main goal during the optimization procedure was to increase the size and thickness and reduce the branching of the crystals. The first two aims were not achieved as the biggest crystal obtained was 0.05 × 0.05 × 0.2 mm, although this proved to be quite adequate. Crystals of DS2–3R have the habit of hexagonal rods which are sometimes hollow. The crystals used for data collection were grown at 302 K, using 2 µl of enzyme at 15 mg ml^{-1} added to 2 µl of the reservoir precipitant which comprised 2.2 *M* NH_4SO_4 with 20 m*M* citrate at pH 5.6.

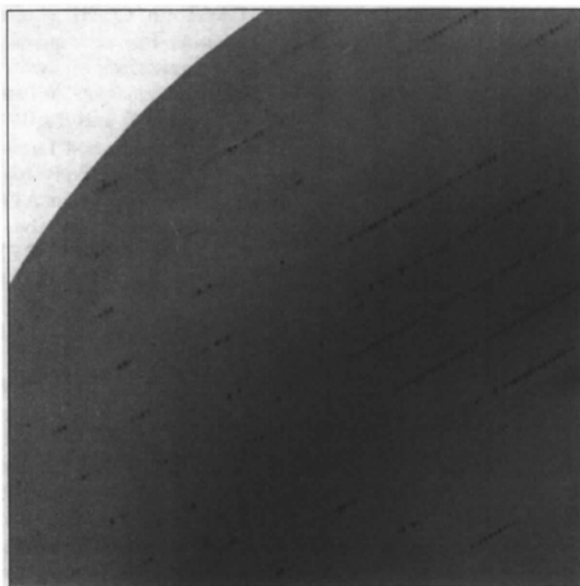

Fig. 1. A 0.4° oscillation image from a flash-frozen crystal of DS2–3R citrate synthase. Diffraction at the edge of the image corresponds to 2.09 Å resolution.

Data were initially collected at 100 K on an in-house X-ray source from a crystal which had been briefly dipped into the crystallization buffer containing 45%(*v*/*v*) glycerol as a cryoprotectant, with freezing conditions derived from Garman & Mitchell (1996). The unit cell was determined to be $a = b = 70.8$, $c = 307.8$ Å, and the space group to be either $P6_122$ or $P6_522$. The large *c* axis limited the resolution collected in-house on a 150 mm radius Mar image-plate detector, and only a 3.9 Å data set was able to be measured. Higher resolution data were obtained on station X11 at DESY, Hamburg, using radiation of wavelength 0.909 Å, again from a flash-frozen crystal. The collimating slits were set at 0.15 mm, and the crystal-to-detector distance to 320 mm, which gave data to 2.09 Å resolution (Fig. 1, Table 1). Data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

Citrate synthase is a dimer of 2 × 42 kDa; however, the V_M value for a dimer is 1.33 Å^3 Da^{-1}, which corresponds to a solvent content of only 7.5% (Matthews, 1968). Assuming one monomer per asymmetric unit gives a V_M of 2.67 Å^3 Da^{-1}, and a solvent content of 54%. The active dimer must therefore be generated by a crystallographic twofold axis. Structure determination using molecular replacement is in progress.

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