Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

β-Carbonic anhydrase from *Pisum sativum*: crystallization and preliminary X-ray analysis

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Received 21 February 2000
Accepted 27 April 2000

Recombinant β-carbonic anhydrase from the garden pea, *Pisum sativum*, was purified to homogeneity and crystallized. Crystals belong to the orthorhombic space group *C*222, with unit-cell parameters a = 136.3, b = 142.5, c = 201.4 Å, $\alpha = \beta = \gamma = 90^\circ$. Crystals typically diffracted anisotropically, with a maximal resolution of 2.0 Å in the strongest direction. The calculated Matthews parameter predicts approximately eight molecules in the asymmetric unit, consistent with previous reports of the molecule being an octamer. However, examination of the self-rotation function revealed no fourfold symmetry axis and multiple weak twofold axes perpendicular to the crystallographic c axis, indicating that the oligomerization arrangement is not that of a 422 octamer.

1. Introduction

Carbonic anhydrases (E.C. 4.2.1.1; CA) are enzymes that catalyse the reversible hydrolysation of CO_2 to bicarbonate. This nomenclature is applied to three different proteins with independent evolutionary origins, designated (in order of discovery) α-CA, β-CA and γ-CA. Although α-CA is best known and most intensively studied in animals (including humans) and β-CA in plants and eubacteria, homologues of all three CAs are found with a very wide phylogenetic distribution: α-CA is found in algae such as *Chlamydomonas reinhardtii*, in angiosperms such as *Arabidopsis thaliana* and in eubacteria such as *Neisseria gonorrhoea*; β-CAs have been found in the archaeote *Methanobacterium thermoautotrophicum*, in fungi including *Saccharomyces cerevisiae* and in the nematode *Caenorhabditis elegans*. γ-CA, first reported in 1994, while having only been formally shown to possess catalytic activity in *Methanosarcina thermophila*, has homologues in both eubacterial and angiosperm species (Alber & Ferry, 1994).

Two main roles have been proposed for β-CA in higher plants as a facilitator of high turnover in the photosynthetic dark reactions. Firstly, since bicarbonate has a higher diffusion coefficient in solution while CO_2 crosses membranes more efficiently, fast interconversion between the two accelerates the travel of CO_2 between the stomatal air cavities and the site of CO_2 fixation. Secondly, at least in C_3 plants, the presence of β-CA in the Calvin cycle enzyme complex in the chloroplastic stroma allows the replenishing of CO_2 near Rubisco, the site of CO_2 fixation (Jebanathirajah & Coleman, 1998). The importance of these two roles is underscored by the fact that in C_3 plants β-CA constitutes 0.5–2% of all leaf protein (Badger & Price, 1994). Overexpressing antisense β-CA mRNA results in plants that are viable at ambient levels of CO_2, but intracellular levels of CO_2 are reduced and a compensatory increase in stomatal conductance can result in larger rates of water loss under stress conditions (Majeau *et al.*, 1994). β-CA, localized in the cyanobacterial carboxysome, has been shown to be an essential component of this process as it catalyses dehydration of the intracellular HCO_3^- pool for the provision of CO_2 for Rubisco (Fukuzawa *et al.*, 1992).

Study of the biochemistry and mechanism of β-CA has benefitted greatly from the fact that most of the required experimental techniques and mechanistic models have already been developed over the course of three decades of intensive studies into the structure and mechanism of α-CA. These studies have revealed a number of intriguing parallels: both enzymes operate at near-diffusion-controlled rates, have proton transfer as the rate-limiting step and use zinc as an essential active-site metal ion (Liljas *et al.*, 1994; Lindskog, 1997). These observations suggest a close convergence between the two enzymes. However, whereas in the case of α-CA the availability of the X-ray crystallographic structure since 1972 (Liljas *et al.*, 1972) has allowed the mechanism to be worked out in some detail through a series of studies including site-directed mutagenesis and structural studies on different inhibitor-bound forms (Liljas *et al.*, 1994; Lindskog, 1997), the lack of structural information for β-CA has hampered efforts to understand the mechanism in more detail.

Here, we present the crystallization and preliminary diffraction analysis for the recombinant pea (*P. sativum*) β-carbonic anhydrase.

2. Methods and results

2.1. Expression and purification

Protein was heterologously produced in *Escherichia coli* BL21(DE3) cells using the pCA plasmid (Provart *et al.*, 1993) using a variant of the protocol of Provart (1993). Cells were grown at 310 K in rich 2xYT media (5 g NaCl, 16 g Tryptone and 10 g yeast extract per litre media) to an A_{600} of 0.7 and were then induced with 0.5 m*M* IPTG (Aldrich) and grown overnight. Cells were then centrifuged for 25 min, washed in 25 m*M* Tris–SO_4 pH 7.8 and lysed by two passes through a French press in the presence of DNAase A (Boehringer Mannheim). After centrifugation, the lysate was brought to 25% saturation in ammonium sulfate at 277 K. After equilibration, this solution was again centrifuged, the precipitate was discarded and the supernatant was brought to 70% saturation in ammonium sulfate at 277 K. After centrifugation, the resulting precipitate was redissolved in 25 m*M* Tris–SO_4 pH 7.8, 20 m*M* NaCl, 5 m*M* dithiothreitol (DTT) and dialysed overnight against the same buffer. The dialysed solution was then loaded onto a Q-Sepharose column, washed with two column volumes of the same buffer and then eluted with a gradient of 0–100% 25 m*M* Tris–SO_4 pH 7.8, 1 *M* NaCl, 5 m*M* DTT. Fractions containing β-CA were identified using polyacrylamide-gel electrophoresis, pooled and loaded onto a *p*-aminomethylsulfonalinamide affinity column (Sigma) using 20 m*M* NaCl, 25 m*M* Tris–SO_4 pH 8.3, 5 m*M* DTT. After washing with three column volumes of the same buffer, the column was eluted with 20 m*M* sodium azide, 25 m*M* Tris–SO_4 pH 8.3, 5 m*M* DTT and the fractions containing protein were pooled and concentrated to roughly 50 mg ml^{-1} protein. Inspection using polyacrylamide gel electrophoresis showed the protein to be highly pure.

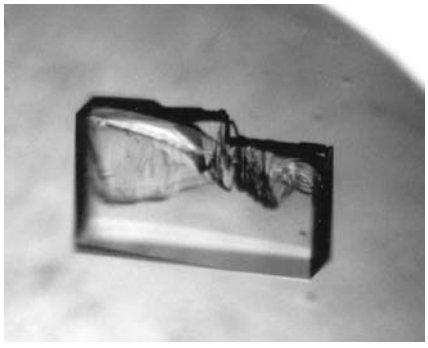

Figure 1
Crystal of β-carbonic anhydrase. The crystal measures approximately 200 × 120 × 80 µm and was grown following the protocol described in the text.

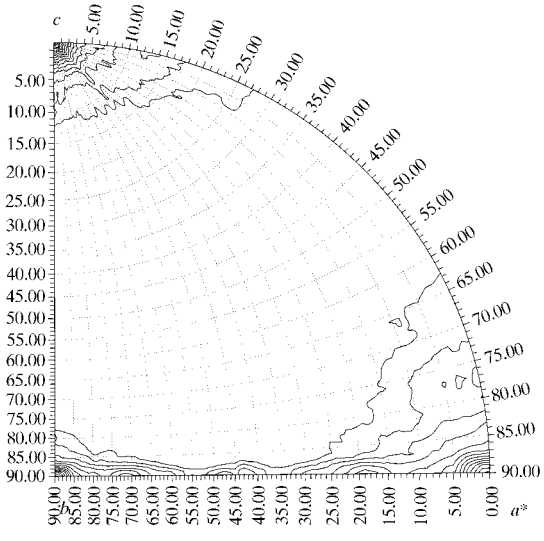

Figure 2
Patterson self-rotation function for the β-CA data set, shown for the $\kappa = 180^\circ$ section. This section demonstrates the existence of a number of weak twofold non-crystallographic symmetry axes orthogonal to the lattice *c** direction. The search was performed using a 25 Å cutoff radius on an origin peak removed Patterson map calculated with reflections in the interval 15.0–4.00 Å using the program *GLRF* (Tong & Rossmann, 1997).

2.2. Crystallization

The protein was crystallized by vapour diffusion against 16% polyethylene glycol (PEG) 4000 (Fluka), 400 m*M* ammonium acetate, 50 m*M* DTT and 100 m*M* sodium citrate pH 5.0 at 277 K. This setup yielded occasional spontaneous crystals of 200 × 100 × 50 µm in size, but routine crystal production required macroseeding (Fig. 1). For data collection, crystals were frozen at 100 K in artificial mother liquor with 25% PEG 4000 and 30% ethylene glycol as cryoprotectant.

2.3. Data collection, processing and analysis

A native data set complete to 2.8 Å was collected at the National Synchrotron Light Source, beamline X8-C using radiation of wavelength λ = 0.9500 Å and a MAR345 area detector. All data were reduced using *DENZO* and scaled using *SCALEPACK* (Otwinowski, 1993) (Table 1). Crystals belonged to the orthorhombic space group *C*222, with unit-cell parameters $a = 136.3$, $b = 142.5$, $c = 201.4$ Å, $\alpha = \beta = \gamma = 90^\circ$. These values are for one high-resolution data set, but unit-cell lengths varied as much as 2% from crystal to crystal. The crystals were generally highly anisotropic in their scattering, with crystals that diffracted to approximately 2.0 Å in the *c** direction but only to approximately 4.0 Å in the *b** directions being common. Self-rotation analysis of the Patterson function was performed using the program *GLRF* (Tong & Rossmann, 1997).

3. Discussion

Although obtaining large visually appealing crystals of β-CA was relatively straightforward, collecting high-quality diffraction data proved considerably more problematic and depended upon a number of changes in the original purification, crystallization and crystal handling protocols. Firstly, while the crystals grow well at room temperature at high PEG 4000 and protein concentrations, smaller but much better ordered crystals grow at 277 K with lower PEG 4000 and protein concentrations. β-CA from plant sources are in general very oxidation sensitive; the addition of DTT throughout the purification proved essential in obtaining protein that crystallized consistently. It was also important to substitute sodium azide for sodium perchlorate in the elution buffer for the *p*-aminomethylsulfonalinamide column, as the latter salt is too strong an oxidizing agent. Experiments where sodium perchlorate was added back to protein purified in azide demonstrated that this salt induces excessive nucleation in sub-millimolar concentrations.

Obtaining good diffraction from frozen crystals was completely dependent upon allowing the crystals to fully equilibrate with the cryoprotectant. Since the crystals are extremely fragile with respect to changes in their solvent environment, obtaining intact cryoprotected crystals required incrementally adding small volumes of the cryoprotectant mix to the edge of the drop at 10 min intervals and then allowing slow equilibration over at least 1 h, with the entire procedure being carried out at 277 K.

Analysis of the packing density shows that 5–12 molecules in the asymmetric unit would yield a reasonable solvent content, with eight molecules being most likely (V_m =

Table 1
Data-collection statistics.

Resolution (Å)	17.0–3.0	3.07–3.00
R_{merge}†	6.4	29.6
$I/\sigma(I)$	11.5	3.9
No. of observations	113757	
No. unique	37371	
Redundancy	3.0	2.6
Completeness (%)	95.2	92.8
Completeness [$I > 3\sigma(I)$] (%)	68.6	38.5

† $R_{merge} = \sum_{hkl}\sum_i |I_i - \langle I\rangle| / \sum_{hkl}\sum_i \langle I\rangle$, where I_i is the ith measurement of the reflection intensity I and $\langle I\rangle$ is the weighted mean of all measurements of I.

2.48 Å^3 Da^{-1}; Matthews, 1968). Since, according to gel-filtration analysis, the reported oligomeric state of the enzyme is an octamer (Björkbacka *et al.*, 1997), we anticipated that self-rotation analysis of the Patterson map would reveal 422 point symmetry as has been reported for electron-microscopy studies of the chickpea enzyme (Aliev *et al.*, 1986). However, the $\kappa = 90^\circ$ slice of the self-rotation function proved to be devoid of peaks, indicating that no four-fold axis of symmetry is present. Since the $\kappa = 180^\circ$ slice had multiple weak peaks orthogonal to the c^* direction, one possibility is that the asymmetric unit contains a pair of objects with 222 symmetry (see Fig. 2). This may be consistent with the report that mutation of the oxidation-sensitive cysteines in *P. sativum* β-CA results in a structural transition from octamer to tetramer, a transition which may mimic the process of enzyme inactivation upon oxidation (Björkbacka *et al.*, 1997). We hope that the ongoing crystallographic analysis will provide us not only with a clearer picture of the chemical mechanism utilized by this enzyme but also with more information on its puzzling oligomeric state.

The authors would like to thank the staff at BNL X8C for assistance with data collection. We are also grateful to Patrick McGinn for purifying the initial batches of the protein and Judith Jebanathirajah for help in maintaining cells. This research was supported by the National Science and Engineering Council of Canada through an Industrial Research Chair to EFP. MSK is the recipient of a Medical Research Council of Canada graduate student scholarship and a University of Toronto Open Scholarship. JRC is funded by a grant from the National Sciences and Engineering Council of Canada.

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