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Crystallization and preliminary X-ray diffraction studies of a β-carbonic anhydrase from the red alga *Porphyridium purpureum*

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The β-carbonic anhydrase from the red alga *Porphyridium purpureum* was heterologously expressed, purified and crystallized. The crystals belong to space group $P2_1$ (unit-cell parameters a = 63.8, b = 113.9, c = 73.8 Å, β = 104.1°) with two subunits per asymmetric unit and diffract to 2.5 Å resolution.

1. Introduction

Carbonic anhydrase (CA; carbonate hydrolyase, E.C. 4.2.1.1) is a zinc metalloenzyme which catalyzes the reversible hydration of CO_2. Based on amino-acid sequence comparison, three evolutionary distinct groups of CAs have been proposed; there is very little similarity (less than 5%) between the three CA families (Hewett-Emmett & Tashian, 1996). The α-CA group includes CAs from higher vertebrates, microalgae and eubacteria; the β-CA group includes CAs from higher plants, algal mitochondrial CAs and eubacterial CAs. The γ-CA group was found in an archaeon, *Methanosarcina thermophila*; however, gene homologues are found in prokaryotes and higher plants. Although X-ray crystal structures of α-CAs and γ-CAs have already been determined (Liljas *et al.*, 1972; Kisker *et al.*, 1996), no β-CA structures have yet been determined. Elucidation of the β-CA structure would provide an interesting example of convergent evolution, showing how the same catalytic activity could have arisen evolutionarily. In a previous report, we isolated and characterized cDNA clones encoding the CA from the red alga *P. purpureum* and revealed that the CA polypeptide is composed of two highly homologous (∼70% sequence identity) halves, each of which corresponds to a β-type CA monomer (Mitsuhashi & Miyachi, 1996). Monomers of other β-type CAs have molecular masses of 24–31 kDa; however, that of *P. purpureum* CA was shown to be 59 kDa. This suggested that the *P. purpureum* CA has arisen by duplication of an ancestral CA gene followed by the fusion of the duplicated CA gene during the process of evolution. This paper describes the crystallization and preliminary X-ray data of β-carbonic anhydrase from *P. purpureum*.

2. Protein expression and purification

Plasmid pET16INT, which expresses a truncated *P. purpureum* CA in which a hydrophobic region at the amino terminus is removed, was constructed by substituting the translational start methionine for Leu75. Two oligonucleotides (primer I, 5′-CAGGAGTCGACCATGGTCAAGCTCGCG-3′ and primer II, 5′-CGAGGATCCGGGTACCATGGTTTTT-3′) and a pUC119 plasmid containing a full-length cDNA of *P. purpureum* CA (gtPCA1, Genbank accession No. D86050) were used to amplify a region encoding a polypeptide (Val76–Gln571) and tagged with *Nco*I sites at both ends. The PCR product was cleaved with *Nco*I and cloned into pET-16b vector (Novagen) previously digested with *Nco*I in a correct orientation to yield pET16INT. The absence of any nucleotide substitution in the cloned fragment was confirmed by DNA sequencing. Competent *Escherichia coli* BL21(DE3) cells were transformed with pET16INT and the transformant was grown at 310 K in Luria–Bertani broth containing 100 µg of either ampicillin or carbenicillin per millilitre and induced (at $A_{600} \simeq 1.0$) with 0.1 m*M* isopropyl thiogalactopyranoside (IPTG) after supplementing the medium with 0.5 m*M* zinc sulfate. After additional growth for 7 h at 299 K, the cells were harvested at 4200*g* and stored at 253 K until use.

All purification steps were carried out at 277 K. The thawed cells were resuspended in 10 m*M* Tris–HCl (pH 9.5) and the suspension was passed through a chilled French pressure cell at 100 MPa. The cell lysate was centrifuged at 15 000*g* for 1 h, following which the supernatant was recentrifuged at 70 000*g* for 1 h. The resulting cell extract was applied to an affinity column (*p*-aminomethylbenzene sulfonamide conjugated Sepharose 6B, Yang *et al.*, 1985), pre-equilibrated with 10 m*M* Tris–HCl pH 9.5. After extensive washing with 5 m*M* Tris–HCl pH 8.5, the CA was eluted with 50 m*M* Tris–HCl pH 8.5 containing 80 m*M* $NaClO_4$. The eluate was dialyzed against 40 m*M* Tris–HCl pH 8.5 and loaded onto an anion-exchange column (Hiload 26/10 Q-Sepharose HP, Pharmacia) which had been previously equilibrated with 40 m*M* Tris–HCl

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Table 1
Intensity data-collection statistics.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	100–2.5 (2.61–2.5)
Observed reflections	90872 (8756)
Independent reflections	32539 (3750)
$I/\sigma(I)$	5.6 (3.5)
Averaged redundancy	2.8 (2.3)
Completeness (%)	94.4 (87.8)
$R_{\rm merge}$† (%)	10.7 (29.7)

† $R_{\rm merge} = \sum |\langle I(h)\rangle - I(h)| / \sum I(h)$.

pH 8.5. The column was washed with 40 m*M* Tris–HCl pH 8.5 and then subjected to a linear gradient (1.2 l) of 0–1 *M* NaCl in 40 m*M* Tris–HCl pH 8.5. The peak fractions of CA, which eluted between 120 and 130 m*M* NaCl, were pooled and dialyzed against 20 m*M* NaCl/20 m*M* Tris–HCl pH 8.5 (complete removal of NaCl caused partial protein aggregation). After being concentrated in a stirred cell using a PM10 membrane (Amicon), the purified enzyme was stored at 277 K until use.

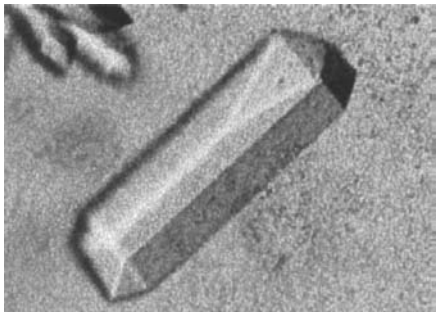

Figure 1
A crystal of the carbonic anhydrase from *P. purpureum*, with maximum dimensions 0.2 × 0.1 × 0.05 mm.

3. Crystallization and preliminary X-ray examination

The hanging-drop vapor-diffusion method was used to crystallize *P. purpureum* CA, wherein 5 µl of the protein solution (30 mg ml^{-1} in 20 m*M* NaCl, 20 m*M* Tris–HCl pH 8.5) was equilibrated against 5 µl reservoir solution (24% polyethylene glycol 4000, 300 m*M* ammonium sulfate, 50 m*M* sodium cacodylate pH 6.75) at 293 K. Plate-shaped crystals were obtained in 2–3 weeks; however, they were thin and partially attached to one another. One of the crystals was placed in the reservoir solution, crushed to a fine powder and used as seeds for further crystal growth. After seeding, crystals (Fig. 1) with maximum dimensions of 0.2 × 0.1 × 0.05 mm grew in 1–2 d.

Preliminary X-ray diffraction experiments were performed using a DIP 2000 (MacScience) image-plate with a Rigaku RU-200 rotating-anode X-ray generator operating at 40 kV and 100 mA. Other experimental conditions were as follows: crystal-to-detector distance, 15 cm; oscillation angle, 1.5°; exposure time per frame, 30 min; temperature, 293 K. Crystals of *P. purpureum* CA were found to belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 63.8 (0.2), b = 113.9 (0.3), c = 73.8 (0.4) Å, β = 104.1 (0.1)°. The molecular weight of the monomer was calculated to be 54 900 Da. The CA exists as a dimer in solution based on gel filtration (unpublished results). On the assumption that there are two monomers in the asymmetric unit, the ratio of volume to unit protein mass (V_m) was calculated to be 2.4 Å^3 Da^{-1}, which is in good agreement with V_m values known for other proteins (Matthews, 1968). The solvent content ($V_{\rm sol}$) was estimated to be 48%. The crystals diffract to 2.5 Å resolution when exposed to rotating-anode X-ray radiation. Intensity data are summarized in Table 1. One heavy-atom derivative was prepared by soaking native crystals in a reservoir solution containing 0.6 m*M* $Na[Au(CN)_4]$ for 12 h. This derivative diffracts to 3.0 Å resolution and is isomorphous to the native. In order to determine the three-dimensional structure, further data collection and structure determination are currently in progress.

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