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# Crystallization and preliminary X-ray diffraction studies of arginase from a thermophilic organism, *Bacillus caldevelox*.

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## Abstract

A thermostable hexameric arginase purified from the extreme thermophile *Bacillus caldevelox* has been crystallized from Hepes buffer at pH 7.5 in the presence of 12% polyethylene glycol 4000 and 10% isopropanol, and from cacodylate buffer at pH 7.2 in the presence of 15% isopropanol and sodium citrate. The latter crystals are more suitable for X-ray diffraction analysis. The crystals are in the orthorhombic space group $P2_12_12_1$ with unit-cell dimensions $a = 156.3$, $b = 148.0$ and $c = 85.4$ Å. The asymmetric unit contains one hexamer (approximate molecular mass 183 kDa) and has a solvent content of approximately 54%. The crystals diffract to 2.8 Å resolution.


## Introduction

Arginase (L-arginine amidinohydrolase, E.C.3.5.3.1) is a metal ion-activated enzyme that catalyses the hydrolysis of L-arginine to L-ornithine and urea as the first step of arginine catabolism in a limited number of bacteria. Bacterial arginases are expressed under aerobic growth conditions and have only been detected in aerobes and facultative anaerobes (Cunin, Glansdorff, Piérard & Stalon, 1986). The enzyme has also been isolated from fungi (Borkovich & Weiss, 1987), plants (Boutin, 1982; Martin-Falquina & Legaz, 1984), and the liver of ureotelic organisms (Reddy & Campbell, 1970) but the most thoroughly characterized arginases are those purified from the liver of ureotelic animals (Kanyo, Chen, Daghigh, Ash & Christianson, 1992), in which the enzyme is one of four comprising the urea cycle, and yeast arginase (Green, Eisenstein, McPhie & Hensley, 1990).

All arginases studied to date require divalent cations for activity. $Mn^{2+}$ is the physiological cofactor, but activation by $VO^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$ and $Cd^{2+}$ has also been reported (*e.g.* Tarrab, Rodriquez, Huitron, Palacios & Soberon, 1974), and under appropriate conditions the trivalent species $V^{3+}$, $Mo^{3+}$ and $La^{3+}$ will also activate some arginases purified from thermophilic bacteria (Patchett, 1988). However the function of the metal ion(s) in the structure and the catalytic mechanism of arginase are unknown, although work with the *Saccharomyces cerevisiae* enzyme has suggested one catalytic $Mn^{2+}$ and one structural metal ion, either $Mn^{2+}$ an $Zn^{2+}$, per subunit (Green, Ginsburg, Lewis & Hensley, 1991).

Arginases can be divided into two groups on the basis of subunit structure. Arginases from a number of bacteria, fungi and plants are homomeric hexamers (Boutin, 1982; Borkovich & Weiss, 1987; Schrell, Alt-Mœrbe, Lanz & Schroeder, 1989), whereas yeast and the mammalian enzymes are homomeric trimers (Brusdeilins, Kuhner & Schumacher, 1985; Green, Eisenstein, McPhie & Hensley, 1990; Kanyo, Chen, Daghigh, Ash & Christianson, 1992). The arginase from the extremely thermophilic bacterium *Bacillus caldevelox* (Deutsche Sammlung von Mikroorganism 411) has been purified and characterized and was found to be a hexamer with at least one $Mn^{2+}$ ion bound per subunit. The quaternary structure was maintained after removal of all catalytic $Mn^{2+}$, but below pH 4 the enzyme dissociated reversibly into subunits. In the presence of $Mn^{2+}$ the *B. caldevox* arginase is highly thermostable, with a half-life for activity of 105 min at 368 K (Patchett, Daniel & Morgan, 1991).

The wide range of metal ions able to substitute for the $Mn^{2+}$ cofactor of arginase make the enzyme an attractive target for the study of metal ion-mediated catalysis, and also of the stabilization of quaternary and tertiary structure by metal ions and the structural basis of enzyme thermostability in general. Crystallization trials of *B. caldevelox* arginase were initiated with a view to providing a structural foundation for such work. This research complements studies by other workers on the trimeric rat liver arginase (Kanyo, Chen, Daghigh, Ash & Christianson, 1992).

## Materials and methods

*B. caldevelox* arginase was purified by a modification of the previously reported method (Patchett, Daniel & Morgan, 1991). $Mn^{2+}$ (10 m*M*) was added to the dialyzed ammonium sulfate pellet and the solution was heat treated at 363 K and pH 7 for 20 min. The phenyl-Superose steps were omitted; instead the final purification step was a second Mono-Q fast protein liquid chromatography (FPLC) run with 50 m*M* L-arginine present in the running buffers. The arginine specifically eluted the arginase earlier in the salt gradient. These modifications eliminated a minor contaminant, of molecular mass 27 kDa, present in earlier preparations. To maintain optimal activity, the enzyme was stored in a 5 m*M* Hepes buffer at pH 7.5, containing 0.2 m*M* $MnCl_2$ and 1 m*M* aspartic acid ('solution buffer').

Arginase was crystallized using the hanging-drop method. An initial screen of the 'solution buffer' against a sparse-matrix crystallization screen covering a range of pH, buffers, precipitants and salt additives (Jancarik & Kim, 1991), revealed 13 solutions which gave crystals or precipitates in the absence of protein (most of these were either sulfate- or phosphate-containing buffers; the latter would have precipitated $Mn^{2+}$). An 8 mg ml$^{-1}$ solution of arginase in the 'solution buffer' was then screened against the remaining 33 solutions using 1 µl drops consisting of 0.5 µl of the arginase diluted with the Hepes buffer (the final protein concentration in the drops was 4 mg ml$^{-1}$). Four conditions gave promising crystals. In the initial experiments crystal growth was very rapid (a matter of hours at 277 K) and resulted in numerous poorly formed

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microcrystals in which growth at the crystal edges exceeded growth at the centre. To overcome this, the protein solution in the drops was diluted up to fivefold with 'solution buffer'.

## Results

Initially, diffraction-quality crystals were obtained from 0.1 *M* Hepes, pH 7.5, 12% polyethylene glycol (PEG) 4000 and 10% isopropanol (IPA), containing 0.2 m*M* $MnCl_2$, 0.5 m*M* aspartic acid and 0.1% sodium azide. The drop sizes varied between 2 and 10 µl and final protein concentrations varied between 1.3 and 4 mg ml$^{-1}$. The crystals had rectangular faces and a rhomboid cross-section, with typical dimensions of 0.3 × 0.1 × 0.1 mm. They proved to be extremely sensitive and had to be mounted in siliconized capillaries directly from the hanging drop, as the addition of well solution caused cracking in the majority of the crystals. This is a problem we have encountered in other crystallization experiments involving combinations of PEG with alcohols, and appears to be due to a failure to reach a correct equilibrium between drop and well solution. These crystals were anlayzed with a Rigaku R-AXIS II imaging-plate detector mounted on a Rigaku RU200 rotating anode X-ray generator operating at 50 kV and 100 mA. Three still images at 45° intervals, indexed using the method of Higashi (1989), gave an orthorhombic unit cell with refined dimensions $a = 156.3$, $b = 148.0$ and $c = 85.4$ Å, and showed that diffraction extended to at least 2.8 Å resolution.

Crystals which were more easily handled were subsequently obtained from 0.1 *M* sodium citrate, 0.2 *M* cacodylate, pH 7.2, 15% IPA, containing 0.2 m*M* $MnCl_2$, 0.5 m*M* aspartic acid and 0.1 m*M* sodium azide. These crystals were morphologically similar to those grown from the PEG/IPA mixture, and had identical unit-cell dimensions. X-ray diffraction data for native arginase were collected from these crystals at room temperature on the R-AXIS using the oscillation method (Arndt & Wonacott, 1977). Two crystals, mounted about the $a^*$ axis, were used to collect a 3.0 Å resolution data set. An oscillation range of 2° was used, with a crystal-to-film distance of 135 mm, and each imaging plate was exposed for 30 min. (Fig. 1). The imaging plates were processed using the program *DENZO* (Otwinowski, 1986) and scaled using *ROTAVATA* and *AGROVATA* (Collaborative Computational Project, Number 4, 1994). The $R_{merge}$ for the data was 6.2% on intensities, giving 39 710 independent reflections (98% complete to 3.0 Å). No cut-off for weak data was used. In the highest resolution shell (3.0–3.1 Å) the $R_{merge}$ and $I/\sigma$ values were 25.6% and 2.1, respectively. Based on systematic absences, the space group was determined to be $P2_12_12_1$. Assuming six molecules of 31 kDa each in the asymmetric unit, the packing volume, $V_m$, was determined to be 2.70 Å$^3$ Da$^{-1}$, giving an estimated solvent content in the crystals of 54% (Matthews, 1968). This suggests that the asymmetric unit contains a complete arginase hexamer.

A search for suitable heavy-atom derivatives is proceeding with the latter crystals, as they were found to be much more stable than those from PEG/IPA, and could be transferred into synthetic mother liquor without cracking or dissolving.

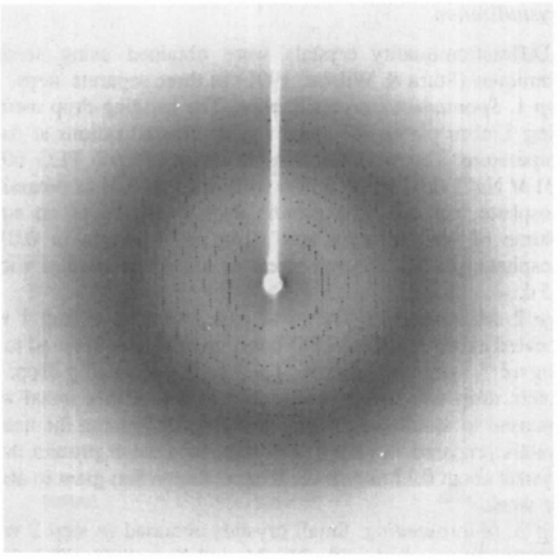

Fig. 1. 2.0° oscillation photographs for *B. caldevelox* arginase. Measurable diffraction data extend to at least 3.0 Å resolution.

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