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FOR THE RECORD

Crystallization and preliminary X-ray diffraction studies of α-amylase from the antarctic psychrophile *Alteromonas haloplanctis* A23

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Abstract: A cold-active α-amylase was purified from culture supernatants of the antarctic psychrophile *Alteromonas haloplanctis* A23 grown at 4 °C. In order to contribute to the understanding of the molecular basis of cold adaptations, crystallographic studies of this cold-adapted enzyme have been initiated because a three-dimensional structure of a mesophilic counterpart, pig pancreatic α-amylase, already exists. α-Amylase from *A. haloplanctis*, which shares 53% sequence identity with pig pancreatic α-amylase, has been crystallized and data to 1.85 Å have been collected. The space group is found to be $C222_1$ with a = 71.40 Å, b = 138.88 Å, and c = 115.66 Å. Until now, a three-dimensional structure of a psychrophilic enzyme is lacking.

Keywords: α-amylase; extremophiles; psychrophilic enzymes; X-ray crystallography

Among the various biochemical drawbacks inherent to life at low temperatures, cold-adapted microorganisms have to cope with the drastic reduction of chemical reaction rates. Enzymes from these psychrophilic microorganisms have evolved toward a high catalytic efficiency in order to maintain adequate metabolic fluxes at often subzero temperatures. According to the current hypothesis (Hochachka & Somero, 1984), this optimization of the catalytic parameters can originate from the highly flexible structure of these proteins, which provides enhanced ability to undergo conformational changes during catalysis at low temperatures. The molecular origin of structure and active site flexibility has been searched tentatively from the primary structure of some psychrophilic bacterial enzymes. All studies point to a low number of potential weak interactions stabilizing the folded conformation (Feller et al., 1996). A critical step will be the availability of the refined crystallographic structure of such enzymes, which presently is lacking, mainly because of difficulties in growing crystals.

Alteromonas haloplanctis is a Gram-negative bacterium collected in Antarctic sea water and secreting a chloride- and calcium-dependent α-amylase (α-1,4 glucan-4-glucanohydrolase, EC 3.2.1.1). Its optimal temperature for growth and α-amylase secretion was found below 4 °C. In addition to a high catalytic efficiency, all biophysical parameters recorded, such as thermal- and guanidinium chloride-induced denaturation, the unfolding of secondary structures or the dissociation constants for chloride and calcium, suggest a loose conformation of the psychrophilic α-amylase. When compared with pig pancreatic α-amylase, the psychrophilic enzyme displays a very low melting temperature (ΔT_m = 20 °C) associated with a reduced conformational stability, $\Delta\Delta G$ = 10 kcal mol^{-1} (Feller et al., 1992, 1994). Interestingly, the primary structure deduced from the cloned gene revealed a striking isology with vertebrate pancreatic and salivary α-amylases. The sequence identity with pig pancreatic α-amylase is 53%, whereas homology between bacterial and vertebrate α-amylases is usually very low. This unexpected property will allow a detailed comparison of the cold-adapted α-amylase with the X-ray structures of pig (Qian et al., 1993) and human (Brayer et al., 1995) pancreatic α-amylases, as well as a fine analysis of the molecular adaptations involved in its structural flexibility.

α-Amylase was purified from culture supernatants of *A. haloplanctis* grown at 4 °C as described previously (Feller et al., 1992), except that the last gel filtration step on Ultrogel AcA54 was conducted in 10 mM Tris, 25 mM NaCl, 1 mM $CaCl_2$, and 1 mM NaN_3 at pH 8.0. The protein was further concentrated to 15 mg/mL for the crystallization experiments.

Preliminary crystallization trials were conducted using the sparse matrix sampling method of Jancarik and Kim (1991). The screening was conducted using the hanging drop vapor diffusion technique with 4-μL droplets equilibrated against 500 μL of reservoir solution at 4 °C as well as 20 °C. This screening, as well as various polyethylene glycol and salt screenings, pointed toward ammonium sulphate and ammonium formate as possible crystallization agents between pH 6 and 7.5. Salt and protein concentration, as

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well as pH, temperature, and additives, were refined as variable parameters. Very tiny needle-shaped crystals were obtained with 0.9–1.1 M ammonium sulphate and 0.1 M Hepes or 0.1 M Tris buffer. Crystals with exactly the same morphology were found when 1.8–2.0 M ammonium formate was used as precipitating agent with Hepes buffer at pH 7.5. These conditions were found at room temperature. Although further optimization of these conditions, together with crystallization experiments in silica gels, was performed, it was not possible to grow crystals suitable for X-ray diffraction studies.

Plate-formed crystals were grown in 60–70% 2-methyl-2,4-pentanediol (MPD) and 0.1 M Hepes buffer at pH 6.8–7.1 at room temperature. The protein to mother liquor ratio was 2:1, and microseeding 24 h after setup of the droplets was necessary in order to obtain single crystals of a reasonable size. Crystals of the size 0.6 mm * 0.3 mm * 0.1 mm were grown within 2 weeks after the seeding. Analyses of aqueous MPD/protein systems have shown that increasing concentrations of MPD have a decreasing effect on the dielectric constant of the medium (Arakawa & Timasheff, 1985). Hereby it was suggested that the decrease in the dielectric constant decreases the interactions of MPD molecules with the protein surface. The fact that such high concentrations of MPD are needed to crystallize the protein may be due to the ability of MPD to induce protein–protein and protein–water interactions, which thereby stabilizes the flexible structure of this protein. Furthermore, most cold-adapted enzymes are characterized by a low global hydrophobicity. The lack of protein denaturation at high concentrations of MPD can possibly be related to this low global hydrophobicity, because MPD binding to solvent-exposed nonpolar regions is disfavored (Arakawa et al., 1990).

Data were collected on a 30-cm MARresearch image plate system, and the X-ray radiation used was CuK_α from a Rigaku RU200 rotating anode operated at 40 kV and 80 mA with a graphite monochromator. Determination of unit cell parameters, as well as integration of reflections, was performed with the program DENZO (Minor, 1993; Otwinowski, 1993) and further data processing was conducted with programs from the CCP4 suite (1994). The space group was unambiguously determined to be $C222_1$ and the refined unit cell parameters are a = 71.40 Å, b = 138.88 Å, and c = 115.66 Å. Assuming a molecular weight of 49,340 Da and one molecule in the asymmetric unit, this gives solvent content of 58% and a volume to mass ratio V_m = 2.91 Å^3/Da (Matthews, 1968). These native data, being 85.9% complete to 1.85 Å resolution, display good statistics, with an R_{merge} of 8.5% based on 153,352 reflections, of which 42,049 are unique. Of the collected data, 79.3% have an $I/\sigma(I) > 2$.

A molecular replacement search using the structure of pig pancreatic α-amylase refined to 2.1 Å resolution (Qian et al., 1993) as a search model, using programs from the X-PLOR package (Brünger, 1993), has failed; thus, a search for heavy atom derivatives will be conducted.

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