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Research paper

Temperature adaptations in psychrophilic, mesophilic and thermophilic chloride-dependent alpha-amylases

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ABSTRACT

The functional and structural adaptations to temperature have been addressed in homologous chloride-dependent α-amylases from a psychrophilic Antarctic bacterium, the ectothermic fruit fly, the homeothermic pig and from a thermophilic actinomycete. This series covers nearly all temperatures encountered by living organisms. We report a striking continuum in the functional properties of these enzymes coupled to their structural stability and related to the thermal regime of the source organism. In particular, thermal stability recorded by intrinsic fluorescence, circular dichroism and differential scanning calorimetry appears to be a compromise between the requirement for a stable native state and the proper structural dynamics to sustain the function at the environmental/physiological temperatures. The thermodependence of activity, the kinetic parameters, the activations parameters and fluorescence quenching support these activity–stability relationships in the investigated α-amylases.

1. Introduction

Life has successfully colonized nearly all environments on Earth, from the permanently frozen polar regions or the arctic permafrost, to the extremely hot deep-sea hydrothermal vents, hot springs or geysers. The range of temperatures compatible with life is quite large and is currently estimated from −20 °C in sea ice [1] to 122 °C in hydrothermal vents [2]. Microorganisms living in these environments are able to cope with the local chemical and physical extreme parameters by various adaptive strategies in order to maintain activity and metabolic functions despite these challenging conditions [3,4]. From an evolutionary perspective, current views suggest that the last universal common ancestor (LUCA) was mesophilic or moderately thermophilic and that extant extremophiles have subsequently colonized harsh environments [5]. However, there are also arguments for a hot origin of life [6,7] and even for a cold origin [8,9]. It is therefore of interest to understand the molecular mechanisms of adaptation to temperature in contemporary enzymes. In a first step towards this goal, we have compared homologous psychrophilic, mesophilic and thermophilic α-amylases. Temperature adaptation has been well studied in thermophiles [10] while this remains fragmental in psychrophiles [11]. Furthermore, comparisons of homologous series of extremophilic and mesophilic proteins are scare [12–16].

α-Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are ubiquitous and widely distributed in microorganisms, plants and animals. These enzymes belong to family 13 in the glycoside hydrolase classification (http://www.cazy.org/and [17]) and catalyze the hydrolysis of internal α(1,4)-glycosidic bonds with net retention of the anomeric configuration in starch, amylose, amylopectin, glycogen and other related polysaccharides through multiple attacks toward the non-reducing ends. As a result of the huge diversity of organisms that synthesize α-amylases, these enzymes exhibit in general a very low degree of sequence similarity, although they adopt the same overall fold [18]. Amongst these enzymes, animal-type α-amylases are homologous enzymes present in all bilaterian animals and in some rare microorganisms, a lateral gene transfer having likely occurred between the two groups [19–22]. All animal-type α-amylases isolated so far display the unusual property to bind a chloride ion at a specific site that induces allosteric activation of the full amylolytic activity. It has been shown that the chloride ion is responsible for the p*Ka* shift of catalytic residues via interactions with active site carboxyl groups [23–25].

Chloride-dependent α-amylases from the Antarctic bacterium *Pseudoalteromonas haloplanktis* and its close homolog from pig

Abbreviations: AHA, α-amylase from *Pseudoalteromonas haloplanktis* (psychrophile); DMA, α-amylase from *Drosophila melanogaster* (ectothermic mesophile); PPA, α-amylase from pig pancreas (homeothermic mesophile); TFA, α-amylase from *Thermobifida fusca* (thermophile); Et-G7-pNP, 4-nitrophenyl-α-D-maltoheptaoside-4,6-O-ethylidene; GdmCl, guanidine hydrochloride; DSC, differential scanning calorimetry; IF, intrinsic fluorescence; CD, circular dichroism.

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(*Sus scrofa*) pancreas have been extensively studied in the context of protein adaptation to low temperatures [26–30]. It was shown that the high specific activity at low temperatures of cold-adapted enzymes is a key adaptation to compensate for the exponential decrease of chemical reaction rates as temperature is reduced. Such high biocatalytic activity arises from the disappearance of various non-covalent stabilizing interactions, resulting in an improved flexibility of the enzyme conformation and in a weak stability [11,31]. Here, we have extended these earlier observations to the chloride-dependent α-amylases from the ectothermic fruit fly *Drosophila melanogaster* and from the thermophilic actinomycete *Thermobifida fusca*. This series covers nearly all temperatures encountered by living organisms (chloride-dependent α-amylases have not been detected in hyperthermophiles). We report a striking continuum in the functional properties of these enzymes coupled to their structural stability and related to the thermal regime of the source organism.

2. Materials and methods

2.1. Gene cloning

The naturally intronless *amy-p* gene (coding for DMA α-amylase) has been originally amplified by PCR from genomic DNA of *D. melanogaster* using the forward primer 5′-AACTTCCATCTG-GAATCATC-3′ and the reverse primer 5′-TGCTCCCCAGCTGTTTAC-3′ and the resulting PCR fragment was cloned in the pGEM-T Easy vector (pGEM-AmyD). To construct an expression vector, restriction sites *ClaI* and *XbaI* were introduced by PCR in 5′and 3′ of the *amy-p* gene, respectively. The pαH12WT* plasmid, modified with an engineered *Nde*I restriction site at the ATG codon and pGEM-AmyD were digested by *Cla*I and *Xba*I and ligated to produce pα-AmyD in which the residual part of the AHA gene was excised by inverse PCR, producing the pAmyD plasmid. Both pAmyD and pET-22b(+) were digested by *Nde*I/*Hind*III restriction enzymes and ligated to give the pET-AmyD plasmid in which the *amy-p* gene is preceded by the signal peptide of AHA. The nucleotide sequence of this construct is given in the Supplementary Fig. S1.

Based on the sequence of *T. fusca* α-amylase Tfu_0985 (GenBank ID: AAZ55023.1), the TFA gene was re-designed in order to replace the native signal sequence by the signal peptide of AHA and to introduce *Nde*I/*Hind*III restriction sites. The codons were optimized for *E. coli* codon usage and this TFA gene was synthesized by GeneArt (Life Technologies) in a pMA vector. PSTFA_pMA plasmid and pET-22b(+) were digested by *Nde*I/*Hind*III restriction enzymes and fragments were ligated to produce the pET-TFA plasmid. The nucleotide sequence of this construct is given in the Supplementary Fig. S2.

2.2. Enzyme production and purification

The recombinant AHA (pαH12WT* plasmid) was expressed in *Escherichia coli RR1* at 18 °C and purified by DEAE-agarose, Sephadex G-100, and Ultrogel AcA54 column chromatography as previously described [32].

The recombinant DMA α-amylase (pET-AmyD plasmid) was expressed in *E. coli* BL21(DE3) at 18 °C in TB (Terrific Broth) containing 100 mg/l ampicillin and α-amylase production was induced by 0.5 mM IPTG (isopropyl β-D-thiogalactoside) at A_{600} of ~4. After 20 h of induction, cells were recovered by centrifugation at 13,000 g for 50 min at 4 °C. Bacteria were disrupted on an EmulsiFlex-C3 homogenizer (Avestin) in the presence of benzonase and protease inhibitors and cell debris were removed by centrifugation. Purification of DMA was achieved in five steps: (a) An ammonium sulfate precipitation at 85% saturation during 1 h at 4 °C. The precipitate was centrifuged at 23,000 g for 50 min and the pellet was dissolved in a minimum volume of Buffer A (50 mM Tris, 1 mM $CaCl_2$, pH 7,5) and then dialyzed against 2 × 2 L of Buffer B (20 mM Hepes, 20 mM NaCl, pH 7,5). (b) A glycogen precipitation in 40% cold ethanol [33]. After dropwise ethanol addition, the insoluble material was removed by centrifugation and glycogen was added to the supernatant. The pellet was solubilized and dialyzed against Buffer A. (c) The solution was loaded on a Q Sepharose Fast Flow anion exchanger (2.5 × 40 cm) and eluted with a linear NaCl gradient (0–1 M) in Buffer A. (d) Fractions displaying amylolytic activity were concentrated to 10 mL by ultrafiltration using a Millipore polyethersulfone membrane (cutoff 10,000 Da) in an Amicon ultrafiltration unit under 3 bars nitrogen pressure and then loaded on a Sephadex G-100 gel filtration column (2.5 × 100 cm) and eluted with buffer A. (e) The fractions of interest were concentrated to 10 mL by ultrafiltration as described above and then loaded on a Ultrogel AcA54 gel filtration column (2.5 × 100 cm) eluted with buffer A. The recombinant TFA α-amylase (pET-TFA plasmid) was produced and purified as described above except that the Sephadex G-100 step was omitted. PPA was from Roche.

Except where specified, enzyme concentration was determined spectrophotometrically at 280 nm using $A^{0.1\%} = 1.90$ for AHA, 1.71 for DMA, 2.41 for PPA and 1.95 for TFA. Dynamic light scattering was performed on a DynaPro NanoStar instrument (Wyatt Technology Corporation) operated in batch mode at 20 °C and fitted with a laser beam emitting at 658 nm. A globular protein model was used for mass estimation. The N-terminal amino acid sequence of DMA and TFA was determined by automated Edman degradation using a pulsed-liquid-phase protein sequencer Procise 494 (Applied Biosytems) fitted with an online phenylthiohydantoin analyzer. Mass determination was performed by ESI-Q-TOF mass spectrometry (Waters, Micromass) in 25% acetonitrile, 0.5% formic acid.

2.3. Enzyme assays and kinetics

α-Amylase activity was recorded using 3.5 mM 4-nitrophenyl-α-D-maltoheptaoside-4,6-O-ethylidene (Et-G7-pNP) as substrate (Infinity™ Amylase kit, ThermoScientific) and by the dinitrosalicylic acid (DNS) method using 1% soluble starch as substrate [23]. Catalytic concentrations of enzymes were determined by the Bradford assay (Pierce).

The effects of pH on amylolytic activity was determined by the DNS method using a poly-buffer containing 25 mM Na acetate, 25 mM HEPES, 25 mM CHES, 25 mM MES and 20 mM NaCl between pH 3.5 −pH 10.5. Chloride-free α-amylase was prepared by extensive dialysis against 20 mM HEPES-NaOH, pH 7,2. The dissociation constants for Cl^- were obtained from the saturation curves as described [23].

Thermodynamic activation parameters were calculated as described [34] using the equations:

$$\Delta G^{\#} = RT \times (\ln(k_B T/h) - \ln k_{cat}) \quad (1)$$

$$\Delta H^{\#} = E_a - RT \quad (2)$$

$$\Delta S^{\#} = \left(\Delta H^{\#} - \Delta G^{\#}\right)/T \quad (3)$$

where k_B is the Bolzmann constant, h the Planck constant, E_a is the activation energy of the reaction and R the gas constant.

2.4. Differential scanning calorimetry

Measurements were performed using a MicroCal VP-DSC instrument at a scan rate of 60 K h^{-1} and under ~25 psi positive cell pressure. Samples (~2 mg/ml) were dialyzed overnight