Biochemical and Biophysical Research Communications 385 (2009) 78–83

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The importance of an extra loop in the B-domain of an α-amylase from *B. stearothermophilus* US100

Bassem Khemakhem [a,b], Mamdouh Ben Ali [a,*], Nushin Aghajari [b], Michel Juy [b], Richard Haser [b], Samir Bejar [a]

[a] Centre de Biotechnologie de Sfax, B.P. 1177, 3018 Sfax, Tunisia
[b] Laboratoire de BioCristallographie, Institut de Biologie et Chimie des Protéines, UMR 5086-CNRS/Université de Lyon, IFR128 "BioSciences Gerland-Lyon Sud", 7 Passage du Vercors, F-69367 Lyon cedex 07, France

ARTICLE INFO

Article history:
Received 20 April 2009
Available online 5 May 2009

Keywords:
α-Amylase
Mutagenesis
Bacillus stearothermophilus
Deletion
Thermostability
Loop
B-domain

ABSTRACT

To provide insight into the potential role of a loop in domain B of several bacterial α-amylases, molecular and structural investigation of *Bacillus stearothermophilus* α-amylase (Amy US100) was used as a model. Combination deletion mutants of G_{213}, I_{214} and G_{215}, described as a loop-forming on the surface bacterial amylases, were subjected to biochemical and structural investigation. Thermoactivity, thermostability as well calcium requirement were studied for each mutant.

Thus, deletion of one residue differently affects only the thermostability. Shortening the loop by deletion of G_{213}–I_{214} or I_{214}–G_{215} improved the thermostability and reduces calcium requirement. However, the deletion of three residues has a negative effect on thermostability and reduces the optimal temperature by 17 °C.

The structural investigation showed that stabilizing deletions contribute to reinforce the architecture of domain B and the active site conformation. The deletion of three residues reduces the flexibility of this region and abolishes a denser hydrogen bond network.

Introduction

Temperature is one of the most important parameters that highly affects enzymes stability and catalytic activity. Thermal unfolding and deamidation of Asn/Gln residues [1,2] are mainly at the origin of thermoinactivation.

One of the most effective approaches to explain thermal adaptation of thermozymes is the structure–function relationship studies. This strategy is based on the opportunity offered by the comparison of homologous enzymes with different thermostabilities. In fact, despite their extremely different thermostabilities, thermophilic enzymes and their corresponding mesophilic often share a high sequence homology, and a relatively similar 3D structure [3–5].

Many experimental approaches have been applied to identify determinants of thermostability. Investigation of this aspect, in different thermo-enzymes, revealed that it seems not to be achieved by a general and universal strategy, but by a combination of individual strategies such as optimized packing of the hydrophobic core, increased number of hydrogen bonds and salt bridges, strengthening of the secondary structures, shortened surface loops and by decreasing the entropy in the denatured state by disulphide bond and proline substitutions [6–8]. It is now widely accepted that most of these stabilizing determinants are associated with a decrease in the structural flexibility [3,9].

α-Amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) are amongst the enzymes of high biotechnological interest that have been studied intensively, both for academic purposes and in particular for industrial uses [10–12].

Various approaches based on rational protein engineering have been applied in order to determine the factors governing the activity dependency on the pH [13], the product specificities [9], oxidation resistance [14] and the origins and the mechanisms of thermostability and thermal adaptation [15–17]. This later aspect has been studied from relationship aspect between enzymatic activity, structural stability, and structural flexibility [10–12]. These studies were mainly focused on domain B and its interface with domain A where the metal and the substrate binding sites are located [18]. In this aspect, several general strategies for increasing the stability of proteins derived from a large number of comparative structural and mutagenesis studies have been applied. One of them was focused on the potential role of a loop in domain B of several bacterial α-amylases. Indeed, Suzuki et al. in [19] proposed that the thermostability of BAA (*Bacillus amyloliquefaciens* α-amylase) was widely increased by the deletion of the equivalent loop formed by R_{176}–G_{177} (BAA numbering). This deletion has been transferred to a number of other bacterial α-amylases derived from various species and similar effects on the

* Corresponding author. Address: Centre de Biotechnologie de Sfax, LEMP, B.P. 1177, 3018 Sfax, Tunisia. Fax: +216 74 440 451.
E-mail address: mamdouh.benali@cbs.rnrt.tn (M.B. Ali).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.04.137

thermostability were observed [9,20–22]. However, no detailed investigations concerning the factors governing the increase in thermostability and the structural repercussion were reported.

This report provides further understanding of the effect of the loop deletion on the thermostabilisation and calcium requirement, taking as model the α-amylase of *Bacillus stearothermophilus* US100 [9,23,24]. Structural features determining the stability are also discussed.

Materials and methods

Media, bacterial strains and plasmids. Media used were Luria broth, Luria agar, Minimal M9 containing 1% (w/v) soluble starch and ampicillin (100 μg/ml). Bacteria were cultured in 500 ml Erlenmeyer flasks, with agitation at 250 rpm, at 37 °C.

Escherichia coli DH5α (F^- ϕ80 Δ*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17 (r_k^-, m_k^+) *deo*R *thi*-1 *sus*E44 λ^- *gyr*A96 *rel*A1) was used as host strain (Invitrogen).

*Escherichia coli XL1-Blue strain: rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *supE44 rel*A1 lac [F' *pro*AB *lacI*^{q} Δ*lac*ZΔM15 *Tn10* (*Tet*^{r})] was supplied with the QuikChange site-directed mutagenesis kit from Stratagene and was used as the host strain for site-directed mutagenesis. The plasmids pMBA19 carrying AmyUS100Δ$I_{214}G_{215}$ was already described [9] while pMBA21-25 carrying AmyUS100 Δ$G_{213}I_{214}$, AmyUS100ΔG_{213}, AmyUS100ΔI_{214}, AmyUS100ΔG_{215} and AmyUS100ΔGIG, respectively, were obtained within this work.

Enzyme assays. The activity assay was performed at optimal temperature (82 or 65 °C) and at pH 5.6 for 30 min. The reaction mixture contained 0.5% (w/v) starch in 25 mM acetate buffer and the enzyme solution in a final volume of 1 ml. The amount of enzyme required to produce reducing sugars equivalent to 1 μmol of glucose per minute was defined as one unit of amylase. The concentration of reducing sugar was determined by the DNS (dinitrosalicylic acid) method [25].

Purification of recombinant amylases, protein quantification and electrophoresis. Purification of AmyUS100 and derived mutants was performed using an IMPACT-CN system from New England Biolabs. The target protein was fused to a tag consisting of the intein and the chitin-binding domain, which allows affinity purification of the fusion precursor on a chitin column. This system utilizes the inducible self-cleavage activity of a protein-splicing element (intein) to separate the target protein from the affinity tag. *E. coli* ER2566 cells containing plasmids pMBA21-25 were induced to an OD of 0.5–0.6 with 0.1 mM isopropyl 1-thio-β-D galactopyranoside and grown overnight at 23 °C. Cells were harvested by centrifugation, resuspended in 20 mM Tris/HCl (pH 8.0), 500 mM NaCl and 1 mM EDTA, and disrupted by sonication in the presence of a mixture of protease inhibitors (Sigma). Debris was removed by centrifugation at 30,000*g* for 30 min at 4 °C, and then the supernatant was applied to a column containing the IMPACT-NT chitin-resin. Self-cleavage of the intein was carried out by overnight incubation with 50 mM dithiothreitol at 4 °C. The protein concentration was determined by the Bradford method [26] using BSA as the standard. Enzymes were separated by SDS/10% PAGE according to the method of Laemmli [27]. Protein bands were visualized by Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) staining. Eluted proteins were pure, as judged by SDS/10% PAGE.

DNA manipulation and mutagenesis. Molecular biological experiments involving plasmid purification, enzyme digestion and modification, and *E. coli* transformation, were performed according to Sambrook et al. [28] or Current Protocols in Molecular Biology [29]. Mutations were introduced using the QuikChange® site-directed mutagenesis kit from Stratagene following the manufacturer's instructions. Different primer was used to delete the G_{213}, I_{214}, G_{215}, $G_{213}I_{214}$, $I_{214}G_{215}$ and $G_{213}I_{214}G_{215}$ residues to construct AmyUS100ΔG_{213}, AmyUS100ΔI_{214}, AmyUS100ΔG_{215}, AmyUS100Δ$G_{213}I_{214}$, AmyUS100Δ$I_{214}G_{213}$ and AmyUS100ΔGIG, respectively. The presence of the appropriate deletions was confirmed by dye terminator cycle sequencing with an automated 373A DNA sequencer (Applied Biosystems Inc.).

Computer-aided 3D-modeling. The automated protein structure homology-modelling Geno3D server was used to generate the 3D model and superposition of the 3D structures was performed with the «rigid» option in the graphics software TURBO-FRODO [30]. Molecular modeling of AmyUS100 was done using the crystal structure of the α-amylase (BSTA) from *B. stearothermophilus* ATCC12980 (pdb accession code 1hvx). Finally, VIEWERLITE™ 5.0 (Accelrys, http://www.accelrys.com/) was used to render figures.

Results and discussion

We have reported, in a previous work, the implication of the I_{214} and G_{215} residues, on the thermostability of AmyUS100 [9]. The structure investigation showed that the I_{214} and G_{215} residues belong to a loop created by five residues R_{212}, G_{213}, I_{214}, G_{215} and K_{216}. This loop occurs between the β11 and β12 and contains two further residues compared to *Bacillus licheniformis* α-amylase, BLA, forming an extra loop.

This part of domain B is very important since it is positioned at the interface with domain A where the metal and the substrate binding sites are located. Related studies were mainly concentrated on deleting two residues issued from primary and/or tertiary structure comparisons [20–22]. So far, no detailed studies

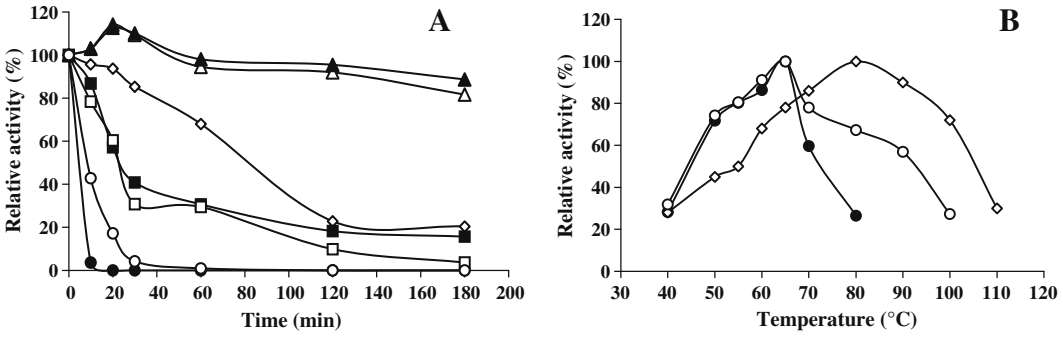

Fig. 1. (A) Comparison between the thermostability of AmyUS100 (◇), AmyUS100ΔG_{213} (■), AmyUS100ΔI_{214} (□), AmyUS100ΔG_{215} (○), AmyUS100ΔGIG (●), AmyUS100Δ$G_{213}I_{214}$ (Δ) and AmyUS100Δ$I_{214}G_{215}$ (▲). The residual activity was expressed in terms of the relative activities after incubation at 90 °C, in the presence of 100 ppm of calcium at pH 5.6. (B) Effect of temperature on the activity of AmyUS100 (◇), AmyUS100ΔG_{215} (○) and AmyUS100ΔGIG (●). The temperature profiles were determined in 50 mM acetate buffer (pH 5.6) at temperatures ranging from 30 to 110 °C. Relative activity is expressed as a percentage of the maximum temperature activity of the enzyme.