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

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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

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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

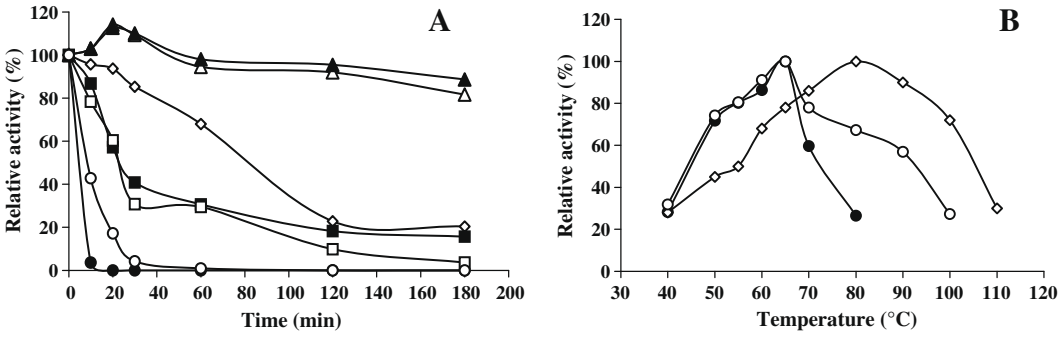

Fig. 1. (A) Comparison between the thermostability of AmyUS100 (◇), AmyUS100ΔG_{213} (■), AmyUS100ΔI_{214} (□), AmyUS100ΔG_{215} (○), AmyUS100ΔGIG (●), AmyUS100Δ$G_{213}I_{214}$ (Δ) and AmyUS100Δ$I_{214}G_{215}$ (▲). The residual activity was expressed in terms of the relative activities after incubation 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.

have revealed the amino-acid residues that should be deleted to enhance thermostability. Furthermore, no detailed investigation concerning the structural features responsible for increasing thermostability of α-amylases, in this region, was reported.

To answer these questions and with the aim of extrapolating the results to other α-amylases, the *B. stearothermophilus* US100 enzyme was taken as a model. This α-amylase shares, in this region, the same sequence and structure arrangement with the majority of α-amylases having the concerned extra loop.

Thus, to undertake a comprehensive study, we have shortened this loop by single deletions (G_{213}, I_{214} or G_{215}), double deletions (G_{213}–I_{214} or I_{214}–G_{215}) and triple deletions (G_{213}–I_{214}–G_{215}). Hence, six mutants of AmyUS100 were generated as described in the materials and methods section. The comparison between the wild-type AmyUS100 and all obtained mutants showed the same pH and starch hydrolysis profile (results not shown), but different behaviors were recorded, as concerns the calcium requirement for activity and thermostability, depending on mutants.

Deletion effects on the thermoactivity and thermostability

The thermostability study of different mutants at 90 °C showed that they can be divided in two categories; stabilizing and destabilizing mutants.

The stabilizing mutants, AmyUS100ΔI$_{214}$G$_{215}$ and AmyUS100ΔG$_{213}$I$_{214}$, showed an enhanced thermostability profile. Indeed, these two mutants retain about 80% of their initial activities after 3 h of treatment at 90 °C against only 20% for the wild type (Fig. 1A). Both mutant enzymes have a temperature optimum similar to that of AmyUS100.

Table 1
Effects of EDTA concentration on the activity of AmyUS100, AmyUS100ΔG$_{213}$, AmyUS100ΔI$_{214}$, AmyUS100ΔG$_{215}$, AmyUS100ΔGIG, AmyUS100ΔI$_{214}$G$_{215}$ and AmyUS100ΔG$_{213}$I$_{214}$. Each enzyme (2.0 U/ml) were incubated with EDTA at pH 5.6 in 50 mM acetate buffer at 60 °C for 30 min. the residual activities in the samples were immediately measured under the standard conditions of the enzyme assay. The values shown are percentages of the respective original activities, which were taken as 100%.

EDTA concentration [mM]	0	50	100	200
AmyUS100	100	15.5	13.7	11.7
AmyUS100ΔG$_{213}$	100	11.1	4.7	1.1
AmyUS100ΔI$_{214}$	100	0	0	0
AmyUS100ΔG$_{215}$	100	6.1	3.0	0
AmyUS100ΔGIG	100	5.9	0	0
AmyUS100ΔG$_{213}$I$_{214}$	100	42.7	41.5	41
AmyUS100ΔI$_{214}$G$_{215}$	100	44.7	41.5	40

The remaining mutations are destabilizing at a variable scale, according to the deleted residues. The deletion of G_{213} or I_{214} lead to a moderate thermostability since the half-life at 90 °C decreased from 80 to 22 min (Fig. 1A). The deletion of G_{215} or the three residues G_{213}–I_{214}–G_{215} resulted in a drastic inactivation of the enzyme. Indeed, only 3% and 0% activity, respectively, were retained after 30 min, when incubated at 90 °C, compared to 20% for AmyUS100. The optimum temperature of AmyUS100ΔG$_{213}$ and of AmyUS100ΔI$_{214}$ was not affected while those of AmyUS100ΔG$_{215}$ and AmyUS100ΔGIG decreased by 17 °C (Fig. 1B).

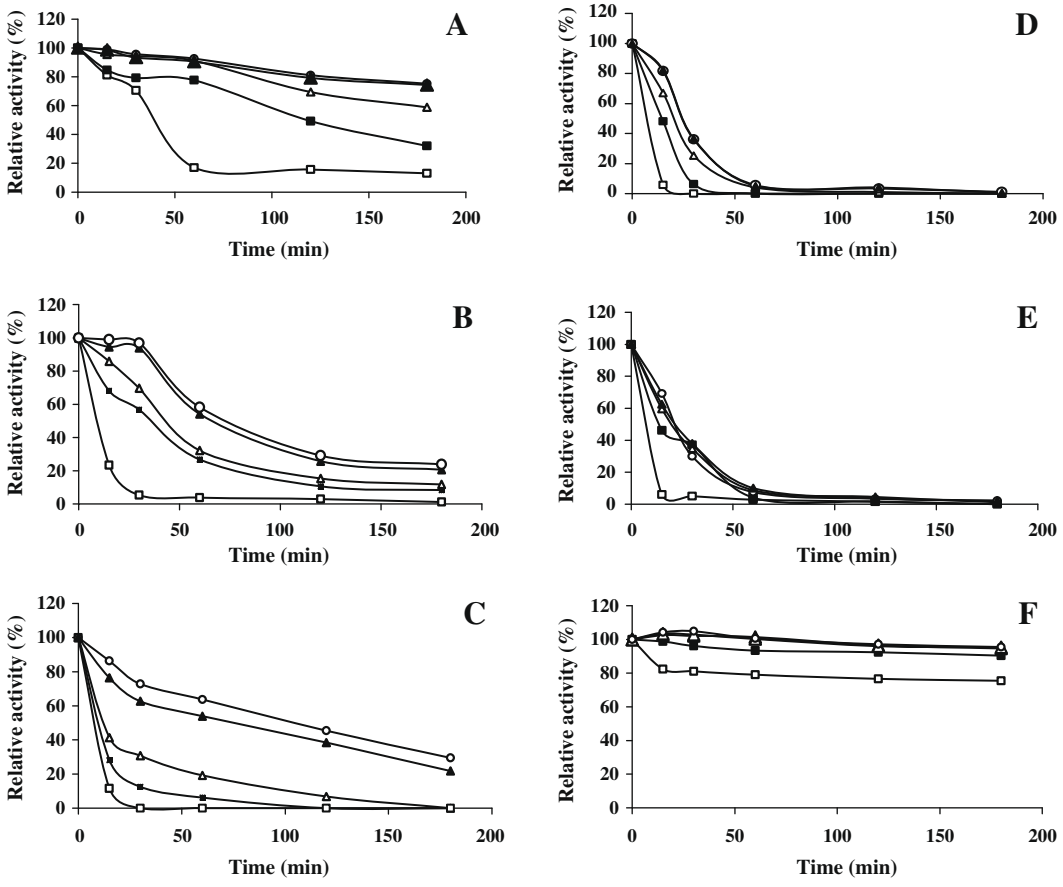

Fig. 2. Effects of different loop deletion on the calcium requirement. Thermostability comparison of AmyUS100 (A), AmyUS100ΔG$_{213}$ (B), AmyUS100ΔI$_{214}$ (C), AmyUS100ΔG$_{215}$ (D), AmyUS100ΔGIG (E), AmyUS100ΔI$_{214}$G$_{215}$ and AmyUS100ΔG$_{213}$I$_{214}$ (F) at 90 °C and pH 5.6 in presence of different calcium concentrations (□): 0 ppm; (■): 25 ppm; (▲): 50 ppm; (Δ): 100 ppm; (○): 200 ppm.

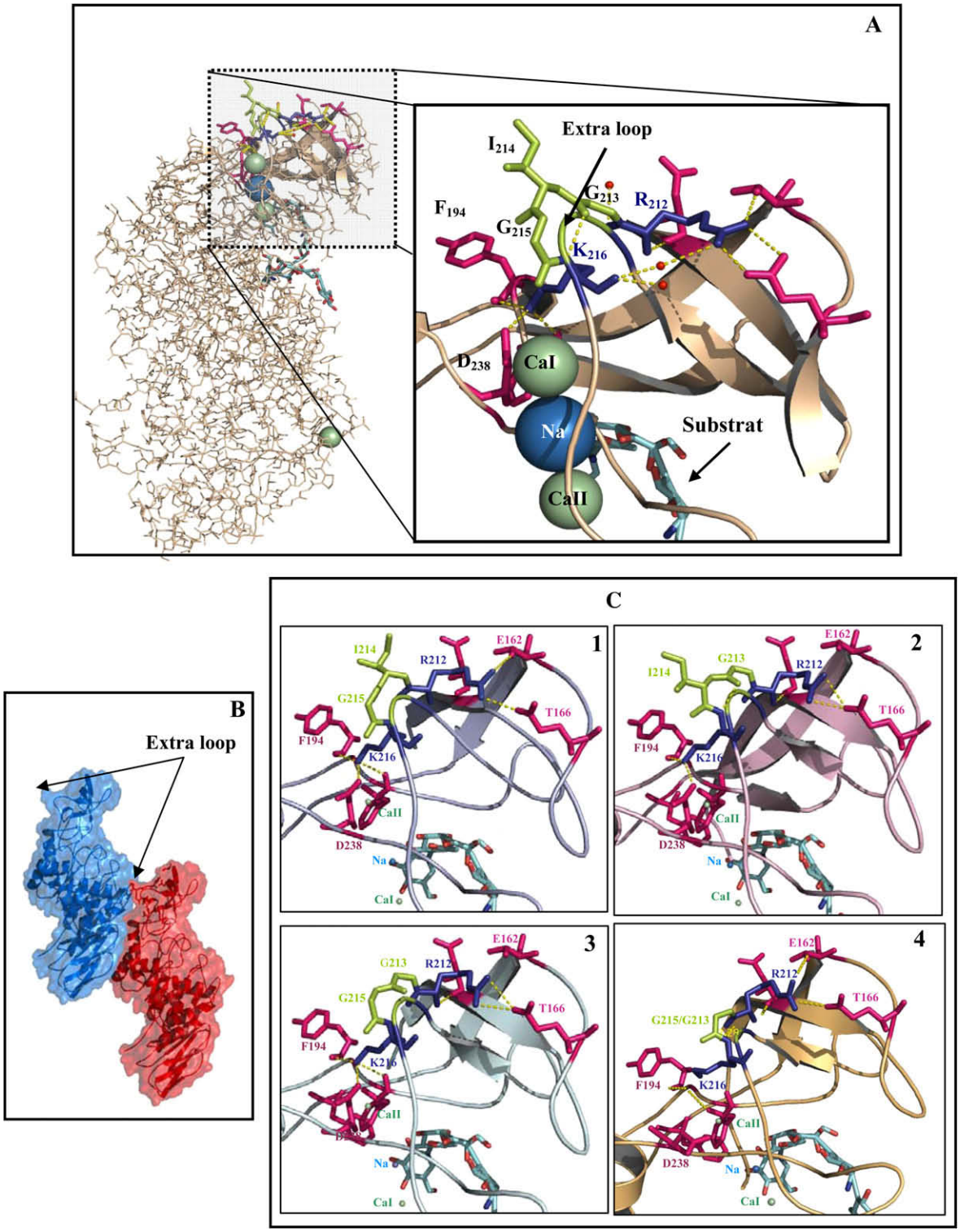

Fig. 3. The structural effects of the different deletions. (A) Domain B focused on the loop region. AmyUS100 (red); calcium ions (green spheres); sodium ion (blue sphere). The substrate analogue (blue stick) occupies the active site. (B) Surface contact between two molecules of AmyUS100 showing the implication of the loop in intermolecular interactions. (C) Structural effects of the different deletion in the loop region: AmyUS100ΔG_{213} (1), AmyUS100ΔG_{215} (2), AmyUS100ΔI_{214} (3), AmyUS100$\Delta I_{214}G_{215}$ and AmyUS100$\Delta G_{213}I_{214}$ (4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Deletion effects on the calcium requirement

The thermostability of the mutants studied was measured in presence of various concentrations of $CaCl_2$. This study showed that the maximum stability of AmyUS100ΔG_{213} and AmyUS100ΔG_{215} were obtained with 100 ppm of calcium (Fig. 2) as already described for AmyUS100 [9]. The deletion of I_{214} increased the calcium requirement since the maximum of thermostability is not obtained even in presence of 200 ppm of $CaCl_2$ (Fig. 2).

Both AmyUS100$\Delta I_{214}G_{215}$ and AmyUS100$\Delta G_{213}I_{214}$ have, in addition to their remarkable thermostability, a low calcium ion requirement, with a maximum of thermostability being obtained

by adding approximately 25 ppm of calcium (Fig. 2). As opposed hereto, the thermostability of AmyUS100ΔGIG mutant cannot be improved even by increasing the calcium ion concentration. This mutant reaches its maximum of stability in presence of 25 ppm of $CaCl_2$.

Deletion effects on the resistance to chelating reagents (EDTA)

Effect of calcium binding on thermal denaturation was investigated at 45 °C in the presence of EDTA. As shown in Table 1, AmyUS100ΔI_{214}, AmyUS100ΔG_{215} and AmyUS100ΔGIG completely lost their activities at a very low concentration of EDTA (50 mM). AmyUS100 and AmyUS100ΔG_{213} enzymes were moderately inhibited by 50 mM EDTA since these amylases retained 24% and 17% of their activities, respectively. However, the deletion of G_{213}–I_{214} or I_{214}–G_{215} increased the resistance to the chelating reagent. For instance, AmyUS100Δ$I_{214}G_{215}$ and AmyUS100Δ$G_{213}I_{214}$ retained 40% of its original activity, when incubated with 200 mM EDTA, compared to 12% for the wild type enzyme.

Structure–function relationships associated with a various deletion

To investigate the structural features determining the stability of the mutants, 3D models of AmyUS100 and its derivatives were constructed on the basis of the crystal structure of the highly similar (97% sequence identity) BSTA [30]. The AmyUS100 model showed a perfect superimposition with BSTA regarding to the very low RMSD (root mean square deviation) value of 0.05 Å.

The AmyUS100 model as well as the 3D structure of BSTA, showed that the loop comprising G_{213}, I_{214} and G_{215} as well as their neighbouring amino acids (R_{212} and K_{216}) seem to be the key players in the stabilization of the α-amylase structure and the calcium ions in this region. K_{216} is crucial for stabilizing the structure by connecting F_{194} and D_{238} (Fig. 3A). These residues participate in the coordination and thus the stabilization of CaI. Thus, the stability of the residue K_{216} seems to be directly related to the stability of the GIG loop. The 3D model of AmyUS100 showed that the internal loop stabilization is the result of two links between the G_{213} and K_{216} (Fig. 3A). Further, the links established by the residues K_{216} and R_{212} are performed with residues implicated in the beginning or ending secondary structures stabilisation such as E_{162} (β8), T_{166} (β9), F_{194} (β10) and D_{238} (β12) (Fig. 3A).

The model of AmyUS100 showed also that the loop (R_{212}–K_{216}) is exposed to the surface and may be implicated in intermolecular interactions, as found in the crystal structure of *B. stearothermophilus* α-amylase [31]. This surface interaction occurs between the loop and the domain A of a close molecule leading to an intermolecular stabilization (Fig. 3B). This surface is stabilized by two connecting points, the first is between I_{213} and I_{400} through hydrophobic links and the second is between Asn_{314} and Ser_{490}. This intermolecular contact surface probably exists also in solution. All of these interactions showed the key role of the loop in structure stability of AmyUS100.

The study of the model of the mutant AmyUS100ΔG_{213} shows that the two connections established by the oxygen of G_{213} with nitrogen of G_{215} and that of K_{216} are abolished. This deletion abolishes also the connection of the NH1 of R_{212} with the OG1 of T_{166} (Fig. 3C1). It involves also a weakening of the connections of coordination of CaI and CaII which can explain the decrease of the thermostability.

Compared to the mutant AmyUS100ΔG_{213}, the deletion of the G_{215} residue causes other effects, such as the loss of the connection of nitrogen of K_{216} and the oxygen of F_{194}. However, a new connection of the oxygen of I_{214} is established with the oxygen of G_{213} rather than nitrogen of K_{216} (Fig. 3C2). The difference recorded in the thermostability profile is probably the consequence of the presence of a nonbulky amino-acid close K_{216} (case of AmyUS100ΔG_{213}) giving it the opportunity to well coordinate the residues implied in secondary structures.

The presence of two adjacent glycine residues (AmyUS100ΔI_{214}) (Fig. 3C3) enhance drastically the flexibility of this region and generate a thermo-sensitive variant with high sensitivity to chelator agents (Table 1).

The deletion of two residues (G_{213}–I_{214} or I_{214}–G_{215}) generated two mutants displaying high thermostability. The deletion allows the connection between the oxygen of R_{212} and nitrogen of K_{216} ensuring a high stability of this area. Moreover, the connections established by R_{212} tend to be reinforced by the creation of a new connection with D_{150} implied in the reinforcement of the stability of the strand β7. All these structural changes offer the possibility to increase the rigidity of the loop. The remaining glycine allows the R_{212} and K_{216} to have a certain flexibility enabling them to make new connections (Fig. 3C4).

The deletion of three residues (GIG) was shown to decrease the thermostability in a compared scale to the deletion of G_{215} residue. AmyUS100ΔGIG was affected by losing of the flexibility due to the absence of glycine and a destabilising steric hindrance of the side-chain of K_{216} and R_{212}. Furthermore, the absence of the I_{214} causes the loss of the inter-molecular connecting point.

Finally, this is the first time that a biochemical and structural study was entirely focused on the extra loop of domain B of *bacillus* α-amylase and related enzymes, taking that of *B. stearothermophilus* as a model. This study confirms previously reported results concerning the enhancement of thermostability obtained by deleting two residues [20–22]. This study did not support the hypothesis that a decrease of structural flexibility is the most important parameter for a good stability (case of G and GIG deletion) [3,9]. Indeed, conformational flexibility is a parameter that has a high impact on protein stability but is, on the other hand, particularly important for catalytic activity. Therefore, one of the key elements for thermal adaptation is a reasonable balance between rigidity and flexibility of the protein structure, well adjusted to the environmental conditions (high temperatures of catalysis).

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