Engineering the thermostability of *Bacillus licheniformis* α-amylase

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DECLERCK, N., MACHIUS, M., JOYET, P., WIEGAND, G., HUBER, R. & GAILLARDIN, C., Engineering the thermostability of *Bacillus licheniformis* α-amylase. Biologia, Bratislava, **57**/Suppl. 11: 203—211, 2002; ISSN 0006-3088.

Bacillus licheniformis α-amylase (BLA) is a highly thermostable enzyme which is widely used in biotechnological processes. Although it is produced by a non-thermophilic bacterium, it remains active for several hours at temperatures over 90 °C under conditions of industrial starch hydrolysis. It is also far more thermostable than the α-amylases from *B. stearothermophilus* and *B. amyloliquefaciens* despite the strong sequence similarities between these three proteins. BLA provides therefore an interesting model for protein engineers investigating enzyme thermostability and thermostabilization. Over the last decade, we have performed an extensive mutational and structural analysis on BLA in order to elucidate the origin of its unusual thermal properties and, if possible, increase its thermostability even further. Before the three-dimensional structure was known, we had used "blind" mutagenesis and identified two critical positions where amino-acid substitutions could either increase or decrease significantly the rate of irreversible thermoinactivation. Once a detailed X-ray structure of BLA was solved, structure-based mutagenesis was used to probe the role of residues involved in salt-bridges, calcium-binding or potential deamidation processes. Our results revealed the key role of domain B and its interface with domain A in determining the overall thermostability of BLA. Most of the mutations we introduced in this region modify the stability in one way or another by influencing the network of electrostatic interactions entrapping a Ca-Na-Ca metal triad at the domain A/B interface. In the course of this mutational study we have constructed over 500 BLA variants bearing single or multiple mutations, among which many were found to be either highly detrimental or slightly beneficial to the stability. The cumulative effect of the mutations enabled us to modulate the enzyme stability over a 50 °C temperature range without perturbing significantly the amylolytic function. Although a full understanding of the origin of BLA natural thermoresistance has not yet been reached, our study demonstrated that

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it is not optimized and that it can be increased or decreased artificially by several means.

Introduction

Because of its remarkably high thermal resistance, *Bacillus licheniformis* α-amylase (BLA) has become the α-amylase most widely used in starch liquefaction processes (VIHINEN & MÄNTSÄLÄ, 1989). For the same reason this enzyme has been extensively studied in both academic and industrial laboratories. The natural thermal resistance of BLA is particularly intriguing given the fact that *B. licheniformis* itself is not a thermophilic microorganism, rather a mesophilic bacterium usually found in temperate soil. Moreover, BLA is far more thermostable than the α-amylase produced by *B. amyloliquefaciens* (BAA) and *B. stearothermophilus* (BStA) (Table 1) even though these three α-amylases present highly homologous primary and tertiary structures (Fig. 1). BLA provides therefore an interesting target for protein engineers not only for improving its industrial performance but also for investigating the molecular basis of enzyme thermostability. Who knows how many hundreds of mutant BLAs have been constructed and characterized in public and private laboratories over the world? Although the wealth of genetic, biochemical and structural data that have been acquired on BLA remains mostly unpublished, the results described in the various papers and patents concerning BLA have demonstrated that this enzyme indeed leaves room for spectacular improvements and scientific achievements (for a review see NIELSEN & BORCHERT, 2000).

This paper relates the story of the protein engineering project that we carried out on BLA thermostability. We have gathered together the results that were obtained in our two laboratories in over a decade of mutational and structural studies on this enzyme. The primary goal of this academic work was to understand the origin of the unusual thermal properties of BLA and in particular to answer the following two questions: 1) what are the special features that render BLA so thermostable compared to its bacterial homologues, BAA and BStA? and 2) is the thermostability of BLA naturally optimized or is it still possible to increase it further by artificial means? Although our work on BLA did not provide complete answers to these questions, it allowed us to reveal several unexpected aspects regarding the thermostability of this amazing α-amylase.

Blind mutagenesis and protein modeling

This engineering project on BLA was initiated in our laboratory after cloning the gene encoding the α-amylase from *B. licheniformis* (JOYET et al., 1984). At that time, little was known on this industrial enzyme and in particular no three-dimensional (3D) structure of BLA was yet available. We knew, though, what the protein looked like since the crystal structure of two eukaryotic α-

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Fig. 1. Primary and secondary structure of *B. licheniformis* α-amylase (BLA) and effect of amino-acid substitutions on BLA thermostability. The complete amino-acid sequence corresponding to mature BLA encoded by the gene cloned by JOYET et al. (1984) is shown in bold. Non-conserved residues in the less thermostable α-amylases from *B. amyloliquefaciens* (BAA) and *B. stearothermophilus* (BStA) are shown below. The four highly conserved regions found in all α-amylases (NAKAJIMA et al., 1986) are boxed. The position of α-helices and β-sheets as determined from BLA crystal structure (MACHIUS et al. 1998) are indicated by full circles and triangles, respectively, colored in blue (domain A), red (domain B) or green (domain C). Black squares indicate the residues binding the three calcium ions. The 29 residues which have been targeted for mutagenesis in our laboratory are colored according to the mutational effects reported in DECLERCK et al. (1990) (position 35, 247, 293, 406 and 450),DECLERCK et al. (1995) (position 133 and 209), DECLERCK et al. (2000) (position 121, 126, 146, 164, 172, 178, 188, 190, 192, 200, 269, 271, 330 and 336) and in a submitted manuscript (position 204, 213, 237, 243, 264 and 265). Amino-acid replacements which have neutral (green), negative (blue) or positive (red) effect on BLA thermostability are indicated in decreasing stabilizing effect above the substituted residues.

Table 1. Properties of several α-amylases from *Bacillus* species.

Source	*B. licheniformis*	*B. amyloliquefaciens*	*B. stearothermophilus*
Number of residues	483	483	515
Identidy with BLA	100 %	81 %	64 %
$T_{1/2}$ (min) at 90 °C [a]	270	2	50

[a] Half-life of the enzymes incubated at 90 °C, pH 6.5, as determined by TOMAZIC & KLIBANOV (1988).

amylases had been solved [Taka-amylase A (MAT-SUURA et al., 1984) and the pig pancreatic α-amylase (BUISSON et al., 1987)] and given the high similarities between the two structures, it was proposed that all α-amylases have the same overall architecture, comprising a central $(\alpha/\beta)_8$ barrel (domain A), a long loop protruding from the barrel (domain B) and a C-terminal Greek key motif (domain C). Later we constructed a model of BLA based on the Taka-amylase A structure (DECLERCK et al., 1995), but even though this model turned out to be of rather good quality, it could not be used for identifying the protein regions or residues determining BLA thermal properties, even less for imagining strategies to increase further the thermostability. Even when the X-ray structure of a protein is available at very high resolution, it is still quite difficult to predict which amino acids are important for thermostability and how to increase it. In fact, if we had known the detailed 3D structure of BLA at the time we started this project, we would probably never have found by rational design the first thermostabilizing mutations we identified following two independent strategies of "blind" mutagenesis.

The first strategy was based on a comparison of the amino-acid sequence and composition of the three homologous bacterial α-amylases, BLA, BAA and BStA. We were hoping to identify some special features in the BLA sequence that could account for the increased thermostability, then test the role of specific residues by mutagenesis. The only relevant difference that we observed in terms of amino-acid composition was an excess of histidyl residues in the BLA sequence and we therefore chose some of these specific histidines as our first targets for mutagenesis (Fig 1). Six histidyl residues (His35, 133, 247, 293, 406 and 450) were replaced by various amino acids using an original method of mutagenesis that had been developed by MILLER (1991). In this method, an amber stop codon is first inserted at the mutation site and the mutated gene is then expressed in 12 different *E. coli* strains containing suppressor tRNAs, each inserting a specific amino acid in response to an amber codon. Thereby, we rapidly created over 100 single or double mutant amylases with defined amino-acid changes that were tested for thermostability *in vitro* (DECLERCK et al., 1990). Most of the substitutions had no significant effect except at the very sensitive position His133 where the mutations either decrease or increase the thermostability. This histidine is replaced by a tyrosine in the less thermostable homologous amylases, BAA and BStA. Unexpectedly, when a tyrosine replaced His133 in BLA, the resulting mutant was not less thermostable, as would have been predicted from sequence comparison, but more thermostable than wild-type BLA. Later, the 19 possible amino-acid exchanges were made at position 133. Ile, Tyr, Met and Val were found to be the most stabilizing, the rate of irreversible thermal inactivation at 80 °C being lowered about 3-fold for the mutants compared to the wild-type enzyme (DECLERCK et al., 1995).

Using classical genetic methods based on random mutagenesis and mutant screening we identified thermostabilizing mutations at a second critical position in BLA. Given the high thermal resistance of the wild-type enzyme, it was difficult to develop an experimental procedure for the screening of more-thermostable mutants. The trick we used was to mutate not the wild-type gene, but a mutant gene encoding a thermosensitive variant. Revertants having increased stability relative to the thermosensitive mutant could thus be screened *in vivo*. By this method, we identified only one stabilizing mutation, replacing Ala209 with a valine. Then by introducing this mutation in the wild-type gene, the resulting A209V mutant exhibited a 3-fold increase of the half-life at 90 °C compared to wild-type BLA (JOYET et al., 1992). By making all the possible substitutions at this position we found that valine was in fact the most beneficial amino acid (DECLERCK et al., 1995). Most surprisingly, it is a valine that is present at this position in both BAA and BStA. Hence at the two critical positions we identified in BLA, 133 and 209, the most stabilizing amino acids are those which are found in the less thermostable homologues, render-

ing the origin of BLA thermostability even more puzzling.

Since the X-ray structure of BLA had not been determined at that time, we used a modeled structure for attempting a structural interpretation of the mutational effects observed at position 133 and 209. This was rather easy in the case of position 209 which is located in the well-conserved domain A. According to our model, Ala209 is at the N-terminal end of helix 3 of the $(\alpha/\beta)_8$ barrel, at the bottom of a small cavity in between helix 3 and 4. Protein modeling showed that this cavity could be filled by introducing small hydrophobic residues such as valine in replacement of Ala209 and we therefore proposed that thermostabilization of BLA at this site was achieved through a cavity-filling effect improving helix packing. Structural predictions were more hazardous in the case of position 133 which falls in the poorly conserved domain B. Moreover, our results of multiple amino-acid replacements at this site had revealed a clear tendency for hydrophobic residues to increase the thermostability but, in our model, His133 was predicted to be on the surface of the molecule. It was thus rather surprising that hydrophobic residues, usually found in protein cores, could increase the stability of BLA if exposed to the solvent. However, secondary structure prediction programs suggested that His133 stands near the N-terminal end of a β-sheet and indeed a good correlation could be drawn between the propensity of the inserted amino acids to form β-sheet and the gain in stability. We therefore proposed that His133 was the first residue of a β-sheet and that BLA was stabilized by the presence of a good β-sheet former at this site (DECLERCK et al., 1995). This proposal, as well as our structural interpretation at position 209, were then confirmed when the crystal structure of BLA was determined (MACHIUS et al., 1995; DECLERCK et al., 1997).

Structure-based mutagenesis and crystallographic studies

Our BLA engineering project entered a new era when the X-ray structure of the protein was solved. Examination of the first BLA structure, obtained in the absence of stabilizing calcium ions, did not allow us to confirm any of the proposals that had been made to explain the naturally high thermostability of BLA (MACHIUS et al., 1995). However, the knowledge of a detailed 3D structure made it possible to investigate more rationally the stability determinants by probing the role of possibly important protein regions and residues.

After careful inspection of the BLA structure we chose 15 new targets for site-directed mutagenesis. Most of the selected residues were located in domain B or its interface with domain A, since we already suspected that this region was crucial for thermostability. There were seven charged residues involved in salt-bridges (Asp121, Arg146, Asp164, Glu271 and Glu336) or in the putative calcium binding site (Asp200 and Asp204), seven Asn/Gln residues (Asn126, 172, 188, 190, 192, and Gln178, 330) which, according to TOMAZIC & KLIBANOV (1988), could be involved in deamidation processes leading to the irreversible thermal inactivation of BLA, and Ala269 which, together with an Arg-Gly deletion at position 178, had been proposed to be responsible for the stability increase of BLA compared to BStA and BAA (SUZUKI et al., 1989).

Each of the 15 selected residues were replaced with various amino acids using the suppressive mutagenesis method we had already employed successfully (see above). A set of 175 new BLA variants bearing single mutations were constructed and characterized *in vitro* (DECLERCK et al. 2000). Most of these variants retained normal amylolytic function, although some of them (at position 164 and 200) had drastically reduced activity. The thermostability of all the active mutants was then tested by measuring their residual activity after incubation at 80 °C. The substitutions appeared essentially neutral (position 146, 178, 271, 330 and 336), always detrimental (position 121, 126, 164, 192, 200, 204 and 269) and sometimes beneficial (position 172, 188 and 190). The most important stabilizing effect was observed at position 190 where the replacement of the original asparagine by a phenylalanine led to a 6-fold increase of the half-life of the N190F mutant compared to that of wild-type BLA. This is the highest increase in stability that we ever obtained as a result of a single mutation in BLA.

Crystallographic studies were then undertaken for BLA variants bearing some of the stabilizing mutations. Unexpectedly, when combining the N190F mutation with the slightly beneficial Q264S/N265Y double mutation we had previously identified (N. DECLERCK, unpublished results), the resulting protein crystallized in the presence of calcium and enabled us to solve the structure of intact BLA at very high resolution (MACHIUS et al., 1998). A very striking feature that this structure revealed was the conserved metal binding site at the interface between domain A and B containing not one calcium ion as in the other amylase structures reported at that time, but two calcium ions and one sodium ion. This metal triad is entrapped
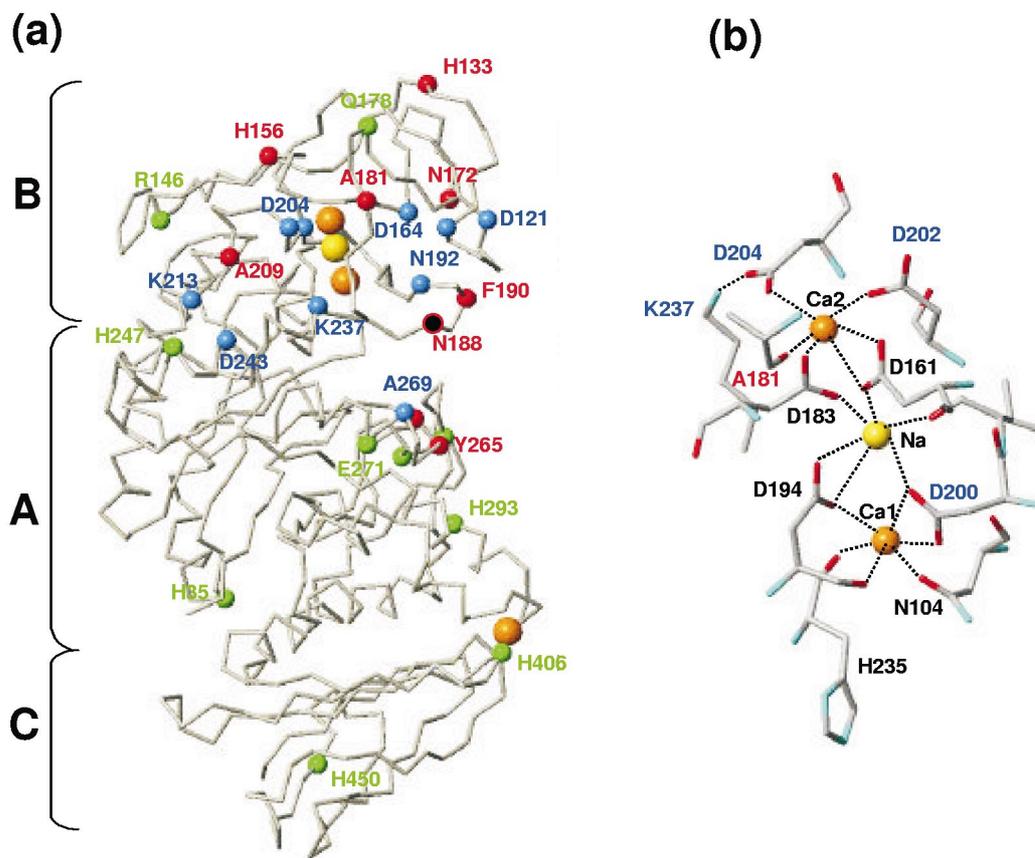
Fig. 2. BLA crystal structure showing the location of the 29 residues targeted for mutagenesis (**a**) and the metal triad binding site (**b**). The 3D structure of BLA is that determined for a thermostable mutant (190F-264S-265Y) that crystallized in the presence of calcium (MACHIUS et al., 1998). Calcium and sodium ions are shown as, respectively, orange and yellow spheres. The mutation sites where amino-acid substitutions have been found to be mostly neutral, always detrimental or sometimes beneficial to BLA thermostability are colored, respectively, in green, blue or red as in Figure 1. Electrostatic interactions within the metal binding site (**b**) are represented as dotted lines.

in an intricate network of electrostatic interactions involving side-chain or main-chain atoms of at least 12 residues from domain A and B. Our mutational study showed the key role of this unusual structural feature. Any mutation we made in the metal liganding cage resulted in the collapse of BLA thermostability. As seen in Figure 2, most of the mutations that change the thermostability in one way or another concentrate in domain B and its interface with domain A where the triadic metal as well as the substrate binding site are located. This region thus appears as the weak part of the protein and any modification of the interaction network may result in a significant decrease or increase in the rate of irreversible inactivation.

Rather surprisingly, four out of the five stabilizing mutations we identified involved the incorporation of hydrophobic residues at the surface and seemed therefore thermodynamically unfavorable. Detailed analysis of the mutant BLA structures enabled us to understand how thermostabilization is achieved and to propose that the mutations might in fact be located at initiation sites for unfolding (MACHIUS et al., submitted manuscript). At position 133, the presence of a valine instead of the original histidine is more favorable for β-sheet formation, thereby releasing local conformational strain on the backbone. Around the Cβ of Ala209 in wild-type BLA, there is a shallow hydrophobic indentation which is smoothed by the mutant valine side-chain, thereby improv-
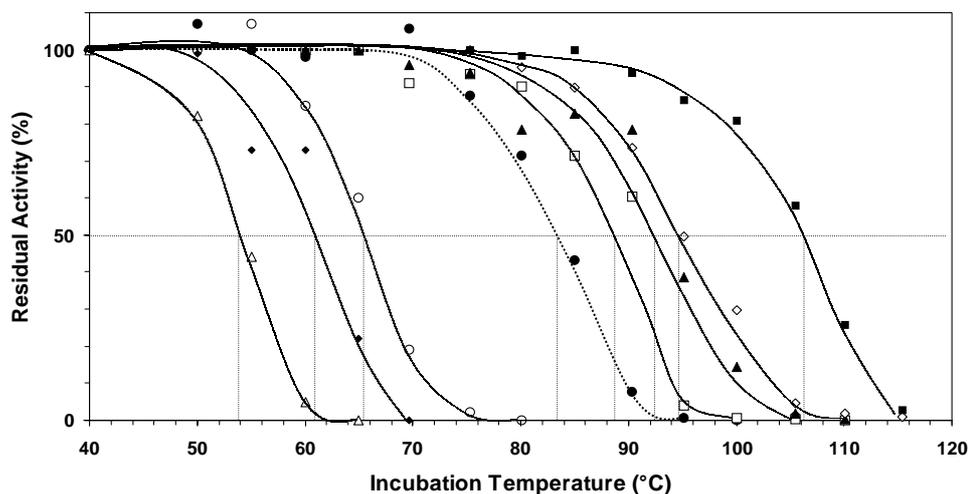
Fig. 3. Cumulative effect of stabilizing and destabilizing mutations in BLA. Curves showing the residual activity of wild-type (●, dotted line) and mutant BLAs after 10 min incubation at various temperatures. The mutations carried by the BLA variants are: D204K-K237D (△), D204K (◆), K237D (○), H133I-A209V (□), H133I-A209V-N190F (▲), H133I-A209V-N190F-Q264S-N265Y (◇), H133I-A209V-N190F-Q264S-N265Y-H156Y-A181T (■). Under the incubation conditions used in the test procedure (Na acetate buffer, pH 5.6, 0.1 mM $CaCl_2$) the temperature of half-inactivation ($T_{50\%}$) is 83 °C for the wild-type enzyme, 106 °C for the most thermostable mutant and 54 °C for the most thermosensitive mutant.

ing helix packing. Residue 190 lies in a long loop that folds over the metal triad and incorporation of a phenylalanine side chain results in the formation of a triple aromatic interaction that might considerably stabilize this loop. Similarly, the mutated side-chain of Tyr265 participates in a complex network of aromatic interactions on the protein surface. In addition, the removal of putative deamidating residues at position 190, 264 and 265 (as well as 172 and 188) may also contribute to reducing the rate of inactivation, although there is no experimental proof that deamidation indeed occurs at any of these sites.

Cumulative effect of stabilizing and destabilizing mutations

A very interesting and useful property of mutations altering protein stability is that their positive or negative effects are often independent and therefore additive. Thanks to this property we could select for stabilizing mutations by screening revertants of a thermosensitive mutant BLA (see above). For protein engineers, the cumulative effect of stabilizing mutations has also allowed augmentation of the rather limited gain in stability usually induced by single mutations. The first two stabilizing mutations we identified in BLA, H133Y and A209V, enhanced the enzyme half-life ($t_{1/2}$)

only 3-fold as single mutations but around 10-fold when combined in the double mutant (JOYET et al., 1992), leading to an increase in the temperature of half-inactivation ($T_{50\%}$) of about 5 °C compared to wild-type (Fig. 3). The thermostability parameters could then be gradually increased by adding more stabilizing mutations. The $t_{1/2}$ and $T_{50\%}$ of the variant combining five mutations obtained in our laboratory (H133I-N190F-A209V-Q264S-N265Y) are, respectively, several hundred times and 12 °C higher than those of wild-type BLA. More recently we have added two other stabilizing mutations identified by others (H156Y, A181T; BISGARD-FRANTZEN et al., 1999) and the $T_{50\%}$ of this seven-fold mutant was increased by about 23 °C compared to wild-type (Fig. 3).

We have also recombined some destabilizing mutations and found that their negative effects were also cumulative (Fig. 3). Both the D204K and K237D mutations involve residues participating in the network of electrostatic interactions entrapping the metal triad. Each of these mutations reduces the $T_{50\%}$ by around 20 °C confirming that the disruption of only one interaction in the metal liganding cage is sufficient to alter drastically BLA thermostability. When combined, these two mutations lead to another 10 °C reduction of the $T_{50\%}$, that is a total loss of about 30 °C compared to wild-type BLA as a result of only two point mu-

tations (N. Declerck, P. Joyet, M. Machius, & C. Gaillardin, submitted manuscript).

Conclusions

Our engineering work on BLA provides unambiguous answer to at least one of the questions that we addressed in the introduction: the natural high thermostability of wild-type BLA is not optimized and it can be further enhanced by protein engineers following both "blind" or "rational" strategies. The fact that BLA is produced by a non-thermophilic organism is probably the reason why its thermostability is not naturally optimized and therefore leaves room for improvement. Nonetheless, we still do not know why *B. licheniformis* possesses such a highly thermostable α-amylase. Resistance to high temperatures could be sporadically required in soil exposed to sunlight or in a fermenting medium, or it could be a side-effect of selection for resistance to other denaturing factors such as proteases or extreme pH.

A very striking and unexpected feature that our crystallographic analysis of BLA revealed was the Ca-Na-Ca binding site at the domain A/B interface. Our mutational studies showed the importance of this metal triad for maintaining the proper folding of domain B and the overall conformation of the active site cleft. However, a similar triadic metal binding site is also present in less thermostable bacterial homologues, as reported recently for BstA (Suvd et al., 2001) and a BAA/BLA chimera (Brzozowski et al., 2000). The enhanced thermostability displayed by BLA can thus not be attributed to the presence of this metal triad. In the BStA structure, however, the network of interactions around the metal ions is slightly different (one interaction involving Asp204 is missing) and this could partly explain the loss of stability. But in the BAA/BLA chimera, the liganding cage made of BAA residues is identical to that seen in BLA. The difference in stability between wild-type BLA and BAA is over 2 orders of magnitude, whilst their amino-acid sequences share over 80% identity. Out of the 483 amino-acid residues composing BLA, besides the Arg-Gly deletion, 91 are substituted in BAA and 133 in BStA. We have exchanged 16 of these non-conserved residues and found only two (Ala209 and Asp121) playing a somewhat moderate role in BLA thermostability. Unexpectedly, two of the mimicking substitutions found in the less-thermostable homologues (H133Y and A209V) together enhanced BLA thermostability by another order of magnitude (Joyet et al., 1992), rendering the differences in stability between the three bacterial amylases even more pronounced while reducing the differences in amino acid sequence. How BLA extra-thermostability was naturally engineered still stands as a fundamental question which has not yet received a complete answer.

How to render BLA even more thermostable is a question, with more applied interests, to which we unexpectedly found multiple answers. Our BLA variants exemplify thermostabilization through better hydrophobic packing and cavity-filling effects, introduction of favorable aromatic-aromatic interactions on the surface, improved formation of secondary structures and release of conformational strain, stabilization of an intrinsic metal binding site, and removal of possibly deamidating residues. By introducing up to seven thermostabilizing mutations into BLA we have enhanced several hundred times the enzyme half-life and increased by over 20 °C the half-inactivation temperature (Fig. 3). From a fundamental point of view, it is interesting to note that the additive effect of a few stabilizing or destabilizing mutations made it possible to engineer the thermostability of BLA over a range of temperature exceeding 50 °C. Moreover, contrary to what is usually observed for naturally- or artificially-thermostabilized enzymes, the specific activity of the highly thermostable variants we constructed was not significantly reduced compared to wild-type BLA.

The mutational work, published or not, performed by other groups has confirmed that there are many ways by which the thermostability of BLA can be enhanced (Nielsen et al., 2000). Interestingly, the engineered hyperthermostable variants of BLA often turned out to be also more resistant to low pH (Shaw et al., 1999). More beneficial mutations have been found and recombined with some of the stabilizing mutations we identified, resulting in highly resistant enzymes with great commercial value (Svendsen et al., 1999). Our engineering work on BLA, performed exclusively with public funds and scientific scope, thus provides a good example of how fundamental research can contribute to improving the biotechnological performance of an industrial enzyme.

INA-PG. The work reviewed in this paper would never have been accomplished without his support and enthusiasm.

References

BISGARD-FRANTZEN, H., SVENDSEN, A., NORMAN, B., PEDERSEN, S., KJÆRULFF, S., OUTTRUP, H. & BORCHERT, T. V. 1999. J. Appl. Glycosci. **46:** 199–206.

BRZOZOWSKI, A. M., LAWSON, D. M., TURKENBURG, J. P., BISGAARD-FRANTZEN, H., SVENDSEN, A., BORCHERT, T. V., DAUTER, Z., WILSON, K. S. & DAVIES, G. J. 2000. Biochemistry. **39:** 9099–9107.

BUISSON, G., DUEE, E., HASER, R. & PAYAN, F. 1987. EMBO J. **6:** 3909–3916.

DECLERCK, N., JOYET, P., GAILLARDIN, C. & MASSON, J. M. 1990. J. Biol. Chem. **265:** 15481–15488.

DECLERCK, N., JOYET, P., TROSSET, J. Y., GARNIER, J. & GAILLARDIN, C. 1995. Protein Eng. **8:** 1029–1037.

DECLERCK, N., MACHIUS, M., CHAMBERT, R., WIEGAND, G., HUBER, R. & GAILLARDIN, C. 1997. Protein Eng. **10:** 541–549.

DECLERCK, N., MACHIUS, M., WIEGAND, G., HUBER, R. & GAILLARDIN, C. 2000. J. Mol. Biol. **301:** 1041–1057.

JOYET, P., GUÉRINEAU, M. & HESLOT, H. 1984. FEMS Microbiol Lett. **21:** 353–358.

JOYET, P., DECLERCK, N. & GAILLARDIN, C. 1992. Bio/Technology **10:** 1579–1583.

MACHIUS, M., WIEGAND, G. & HUBER, R. 1995. J. Mol. Biol. **246:** 545–559.

MACHIUS, M., DECLERCK, N., HUBER, R. & WIEGAND, G. 1998. Structure **6:** 281–292.

MATSUURA, Y., KUSUNOKI, M., HARADA, W. & KAKUDO, M. 1984. J. Biochem. **95:** 697–702.

MILLER, J. H. 1991. Methods Enzymol. **208:** 543–563.

NAKAJIMA, R., IMANAKA, T. & AIBA, S. 1986. Appl. Microbiol. Biotechnol. **23:** 355–360.

NIELSEN, J. E. & BORCHERT, T. V. 2000. Biochim. Biophys. Acta **1543:** 253–274.

SHAW, A., BOTT, R. & DAY, A.G. 1999. Curr. Opin. Biotechnol. **10:** 349–352.

SUVD, D., FUJIMOTO, Z., TAKASE, K., MATSUMURA, M. & MIZUNO, H. 2001. J. Biochem. **129:** 461–468.

SUZUKI, Y., ITO, N., YUUKI, T., YAMAGATA, H. & UDAKA, S. 1989. J. Biol. Chem. **264:** 18933–18938.

SVENDSEN, A., BORCHERT, T. V. & BISGARD-FRANTZEN, H. 1999. Patent WO99/19467.

VIHINEN, M. & MÄNTSÄLÄ, P. 1989. Crit. Rev. Biochem. Mol. Biol. **24:** 329–418.