Kinetic Stabilization of *Bacillus licheniformis* α-Amylase through Introduction of Hydrophobic Residues at the Surface*

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It is generally assumed that in proteins hydrophobic residues are not favorable at solvent-exposed sites, and that amino acid substitutions on the surface have little effect on protein thermostability. Contrary to these assumptions, we have identified hyperthermostable variants of *Bacillus licheniformis* α-amylase (BLA) that result from the incorporation of hydrophobic residues at the surface. Under highly destabilizing conditions, a variant combining five stabilizing mutations unfolds 32 times more slowly and at a temperature 13 °C higher than the wild-type. Crystal structure analysis at 1.7 Å resolution suggests that stabilization is achieved through (a) extension of the concept of increased hydrophobic packing, usually applied to cavities, to surface indentations, (b) introduction of favorable aromatic-aromatic interactions on the surface, (c) specific stabilization of intrinsic metal binding sites, and (d) stabilization of a β-sheet by introducing a residue with high β-sheet forming propensity. All mutated residues are involved in forming complex, cooperative interaction networks that extend from the interior of the protein to its surface and which may therefore constitute “weak points” where BLA unfolding is initiated. This might explain the unexpectedly large effect induced by some of the substitutions on the kinetic stability of BLA. Our study shows that substantial protein stabilization can be achieved by stabilizing surface positions that participate in underlying cooperatively formed substructures. At such positions, even the apparently thermodynamically unfavorable introduction of hydrophobic residues should be explored.

Some general rules for increasing the stability of proteins have been derived from a large number of comparative structural and mutagenesis studies (1, 2). Among the most generally recognized strategies for protein thermostabilization are: increasing the hydrophobic packing in the interior, decreasing surface hydrophobicity, extending networks of salt-bridges and hydrogen bonds, engineering disulfide bonds or metal binding sites, shortening or strengthening solvent-exposed loops and termini, increasing the extent of secondary structure formation, and replacing residues responsible for irreversible chemical alterations of the protein structure. Yet, these general rules may not always be applied successfully and examples of engineered mutations resulting in effects opposite to the ones expected are legion. Such studies have also failed to reveal outstanding features associated with the adaptation of proteins to a given temperature range, i.e. psychrophilicity, mesophilicity, thermophilicity, and hyperthermophilicity. While most natural proteins seem to achieve their respective stability by accumulating a large number of weakly stabilizing interactions that result in a large net effect, some have acquired specialized structural features that cannot easily be transferred in a general way into other proteins (3, 4). Elucidating the origin of thermal stability for a given protein and finding ways to increase it remains a specific and challenging task.

Highly thermostable *Bacillus licheniformis* α-amylase (BLA) is widely used in biotechnology for the initial steps of starch degradation at temperatures up to 110 °C (5). The primary substrate for BLA is starch, but similar compounds, such as glycogen or smaller oligosaccharides, are readily converted as well. Furthermore, BLA, like other α-amylases, shows pronounced transglycosylation activity and is therefore also used in synthetic chemistry for the enzymatic synthesis of oligosaccharides. For this purpose, attempts are being made to modify the enzymatic activity of BLA in order to accommodate a wider variety of substrates with varying specificity (6). The main focus, however, is the search for α-amylases that are less prone to oxidative effects, highly stable at temperature and pH extremes and still enzymatically active. Although thermophilic and acidophilic α-amylases have been found in extremophiles (3, 7), BLA remains a prime target for extensive protein engineering projects (8–10).

When subjected to high temperatures, BLA unfolds irreversibly and precipitates (11, 12). The derivation of comprehensive and accurate thermodynamic parameters for the folding/unfolding process of BLA is therefore not straightforward. Nevertheless, a wealth of information has been acquired in various laboratories providing insights into the origin of BLA stability (11–16). Several hundred BLA variants have been constructed and characterized and have led to the identification of protein regions and residues that are important for thermostability (13, 17–20). BLA is also one of the few proteins for which detailed biochemical studies on the mechanism of irreversible thermal inactivation have been carried out. It is suspected that the main cause of irreversible BLA inactivation at high tem-
Experimental Procedures

Unfolding and Inactivation Kinetics—BLA variants containing various combinations of the mutations, H133V, N190F, A209V, Q264S, and N265Y were constructed as described (18). The thermally induced irreversible unfolding of BLA was followed by circular dichroism (CD) spectroscopy using a Jasco J-715 CD-spectrometer equipped with a peltier thermostating cuvette holder. Samples were incubated at 85 °C in 50 mM sodium acetate, 0.1 mM CaCl₂, pH 5.6 and the CD signal recorded at 222 nm for up to 800 min. The concentration of the samples was routinely around 0.1 mg/ml with a pathlength of 0.1 cm. To investigate the influence of intermolecular interactions on the unfolding behavior, the concentration of wild-type BLA was varied over about three orders of magnitude (0.85 mg/ml with 0.1-cm pathlength, 0.1 mg with 0.1-cm pathlength, and 0.02 mg/ml with 0.2-cm pathlength).

Pseudomelting curves were recorded by heating BLA with a rate of 1/min from 20 to 95 °C and following the CD signal at 222 nm. Because of the extreme thermostability of BLA, it was technically not possible to record the entire unfolding curve. In order to obtain an estimate of the relative stabilities for the mutants, we determined the temperature where unfolding started. After normalizing the raw data for sample concentration, this point was derived by calculating the intersection between the CD baseline at low temperatures (between 20 and 60 °C) and a line drawn through the linear part of the transition region.

Crystallization—The BLA variant containing all five stabilizing mutations was crystallized by vapor diffusion from drops containing 4 μl of protein solution (10 mg/ml in 50 mM Tris/HCl, pH 8.0) plus 4 μl of reservoir solution (50 mM Hepes, 1 mM ammonium sulfate, 1% (v/v) polyethylene glycol (PEG) 5000, pH 7.0) equilibrated against 1 ml of reservoir solution at 20 °C. Hexagonal bipyramids appeared after 3–10 days and grew to a final size of 0.5 mm in length within 1–3 weeks. The crystals show the symmetry of space group P6₁, with cell constants of a = b = 91.3 Å, c = 137.7 Å, and contain one molecule in the asymmetric unit.

X-ray Data Collection, Structure Solution, and Refinement—Diffraction data of up to 1.7 Å Bragg spacing were collected on a MAR-research image plate system using CuKα radiation and a beam-focusing mirror system (MSC). Data were processed and reduced with the DENZO/SCALEPACK package version 1.9.0 (25). Details of the data collection statistics are presented in Table II.

The starting point for refinement was a crystal structure of another BLA variant, which had previously been solved by Patterson search techniques using the cleaved form of BLA (22). All refinement steps were carried out with the program CNS version 1.0 (26). In order to minimize model bias, ten residues around the individual mutation sites were removed from the starting structure, which was then subjected to simulated annealing at a starting temperature of 3000 K, followed by cycles of energy-restrained positional refinement of the coordinates, calculation of individual B factors, as well as visual inspection and manual correction of the model using the program O (27). The missing residues were built at the full resolution, and water molecules added where stereochemically reasonable. Refinement statistics are listed in Table II.

Selection of BLA Mutants—For the present study, we have selected five mutations that we had previously identified as retarding the irreversible thermal inactivation of BLA. The mutation H133V was identified while probing residues that are not conserved between BLA and the highly homologous but
much less stable α-amylase from *B. amylophilica* (17, 18). The mutation A209V was obtained by *in vivo* screening of suppressive mutations in a thermosensitive BLA variant (20). The double mutant Q264S/N265Y was found when replacing suppressive mutations in a thermosensitive BLA variant (20).

The kinetic stability is expressed in terms of the half-life for the thermally induced inactivation at 85 °C (see “Experimental procedures” for details). The shift of the onset of unfolding (ΔOU) is expressed relative to wild-type BLA. The mutations are as follows: 133, H133V; 190, N190F; 209, A209V; 264, N264S; 265, Q265Y. These four mutations were identified before detailed experimentally obtained structural information about BLA was available. Recently, we have carried out a broad study based on the first reported crystal structure of BLA (14) where we probed 15 positions and characterized more than 175 mutations in terms of their influence on the kinetic thermostability of BLA (19). During this study, we identified the mutation N190F as the most stabilizing single site mutation found for BLA so far.

**Thermal Inactivation Behavior of the BLA Variants**—The unfolding kinetics of wild type BLA and BLA variants at 85 °C was followed by recording the CD signal at 222 nm (Fig. 1A). Highly destabilizing conditions (low pH and calcium concentration) were used in order to reduce the incubation time required for accurate measurements. Experiments with different sample concentrations show that BLA unfolding under these conditions is a monomolecular process (11). Overall, the kinetics of BLA unfolding can be modeled with high accuracy with a single exponential term as shown in Equation 1,

\[
N_t = N_0 \exp(-k_{\text{unf}} \cdot t)
\]  

(Eq. 1)

where \(N_t\) specifies the concentration of native molecules at time \(t\), \(N_0\) specifies the concentration of native molecules at time 0, and \(k_{\text{unf}}\) is the apparent rate constant for BLA unfolding.

The half-life for the thermally induced denaturation of wild-type BLA is about 13 min. The five mutations described above all prolong the lifetime of BLA at 85 °C to a various extent (Table I). The stabilizing effects are cumulative with the BLA variant that contains all five mutations exhibiting a half-life of 447 min, which is 32 times longer than the wild type.

The investigated variants not only unfold more slowly at 85 °C, but the onset of unfolding is also shifted toward higher temperatures (Fig. 1B). From pseudomelting curves it can be estimated that the most stabilized BLA variant has a melting temperature that is about 13 °C higher than that observed for wild type BLA (Table I).

**Structure Determination**—We have determined the crystal structure of the BLA variant containing the five stabilizing mutations mentioned above to a resolution of 1.71 Å. Data collection and refinement statistics are shown in Table II. A representative part of the electron density is shown in Fig. 2A.

The overall topology of BLA, shown in Fig. 2B, is typical for α-amylases with a central A domain containing a (βα)_{10}-barrel forming the core of the enzyme. Attached to this core is a C-terminal domain that invariably comprises a Greek key motif. A third domain (B domain) is established as a loop-rich protrusion from the A domain. This domain is the most dissimilar among the known α-amylase structures and varies widely in size, with BLA exhibiting the largest and most complex structure (14, 22).

In the following, we present a structural interpretation of the stabilizing effects exerted by the mutations. A general overview of the location of these mutations is given in Fig. 2.

**Position 133**—Residue 133 is located at the surface of domain B where it is the first residue of the large central β-sheet in this domain (Figs. 2 and 3). The wild-type histidine at this position is part of a weak hydrogen-bonding network (Tyr-175OH ↔ Wat ↔ His-133NE2 and His-133ND1 ↔ Gly-131O) involving a well-ordered water molecule at the bottom of a small surface indentation in between β-strands Bβ4, Bβ5, and Bβ6. The histidine side chain partly fills this cavity and shields the hydrophobic interior of the location of these mutations is given in Fig. 2.

### Table I

| Variant | Kinetic stability | ΔOU |
|---------|------------------|-----|
| Wild-type | 14 | – |
| 264/265 | 19 | 2 |
| 190/264/265 | 71 | 6 |
| 133/190/209 | 384 | 11 |
| 133/190/209/264/265 | 447 | 13 |

The kinetic stability is expressed in terms of the half-life for the thermally induced inactivation at 85 °C (see “Experimental procedures” for details). The shift of the onset of unfolding (ΔOU) is expressed relative to wild-type BLA. The mutations are as follows: 133, H133V; 190, N190F; 209, A209V; 264, N264S; 265, Q265Y.

### Table II

| Crystal parameters, data collection and structure refinement statistics |
|-----------------------------|
| Data collection |
| Space group | P61 |
| Unit cell dimensions (Å) | a = b = 91.3, c = 137.5 |
| Resolution range (Å) | 25.9–1.71 |
| Number of measured reflections | 231592 |
| Number of unique reflections | 61355 |
| Average multiplicity | 3.8 |
| Completeness (%) all data | 83.8 |
| Rmerge |
| 1.88–1.79 Å | 78.8 |
| 1.79–1.70 Å | 25.0 |
| \( R_{merge} \) all data | 0.073 |
| 1.88–1.79 Å | 0.45 |
| 1.79–1.70 Å | 0.31 |
| Refinement |
| Resolution range | 25.9–1.70 Å |
| σ cutoff | 0.0 |
| Reflections used | 58628 |
| Rmerge |
| 27.5–1.70 Å | 0.174 |
| 1.73–1.70 Å | 0.330 |
| \( R_{merge} \) all data | 0.156 |
| 27.5–1.9 Å | 0.319 |
| No. of protein atoms | 3905 |
| No. of metal atoms | 4 |
| No. of water molecules | 324 |

**Ramachandran most favored/additional (%)**

| Overall mean temperature factor (Å²) | 22.84 |
| Ram. deviation of B factors for bonded atoms (Å²) | 1.143 |
| Mean temperature factor for protein atoms (Å²) | 21.51 |
| Mean temperature factor for metal atoms (Å²) | 19.28 |
| Mean temperature factor for solvent atoms (Å²) | 35.99 |
| Ram. deviation from ideal bond lengths (Å) | 0.009 |
| Ram. deviation from ideal bond angles (°) | 1.359 |
| Ram. deviation from ideal dihedral angles (°) | 24.00 |
| Ram. deviation from ideal improper angles (°) | 0.82 |

\[ R_{merge} = \sum \sum | F_{i,j} - | F_{i,j} | | / \sum \sum | F_{i,j} | \]

where the outer sum (\( h \)) is over the outer sum (\( h \)) and \( h \) is the sum (\( i \)) over the set of independent observations of each unique reflection.

### Notes

1. N. Declerck, unpublished results.

2. These four residues specify the concentration of native molecules at time \( t \), \( N_0 \) specifies the concentration of native molecules at time 0, and \( k_{\text{unf}} \) is the apparent rate constant for BLA unfolding.
formed by residues Ala-117, Ile-135, and Tyr-175. It was observed that the introduction of hydrophobic residues at position 133 is particularly effective in increasing BLA stability (18), with isoleucine, tyrosine, and valine being the most stabilizing residues. As a consequence of the H133V mutation, the hydrogen-bonding network that originally involved the histidyl side chain is disrupted, and the hydrophobic nature of the surface indentation is increased. The crystal structure of the mutant reveals that there is a main chain displacement around residue 133 with the \( \phi/\psi \) angles of residue 133 changing from \(-150/163^\circ\) for the histidine in wild-type BLA to \(-135/145^\circ\) for the valine in the mutant (Fig. 3A). The latter values are more favorable for forming the hydrogen-bonding patterns observed in \( \beta \)-sheets. As a result, the hydrogen bonds between the two strands are slightly shorter and consequently the entire \( \beta \)-sheet presumably more stable when compared with wild-type BLA (Fig. 3B). The interactions of the wild-type histidine side chain with its neighbors prevent the side chain from turning more into the hydrophobic indentation causing the main chain to slightly tilt backwards. A detailed analysis of the deviation of geometric parameters, such as bond lengths, dihedral, and improper angles from ideal values (carried out with the program CNS (26)) shows that there is considerable strain around position 133 in wild-type BLA (Fig. 4C). This strain is diminished and spread out over a wider range in the H133V mutant.

**Position 190**—Residue 190 lies at one end of the central cleft between domains A and B with the side chain fully solvent-exposed. It is located in a long loop region that undergoes a disorder-order transition upon metal binding (Fig. 2). The structure of native BLA harboring an asparagine at this position is not known, but the mutational profile provides valuable information on possible thermostability determinants (19). Introducing large and mainly hydrophobic residues increases the stability of BLA by a factor of up to four. Surprisingly though, tyrosine has no effect. On the other hand, introducing small
side chains, e.g. alanine, reduces the stability by a factor of up to four. Introducing a negatively charged residue in the form of a glutamate at position 190 is similarly destabilizing, suggesting that a potential deamidation of Asn-190 is deleterious to BLA stability.

Overall, the preference for hydrophobic residues indicates increased hydrophobic packing with underlying aromatic residues. Indeed, the mutation of Asn-190 to phenylalanine creates a triple aromatic interaction where the ring system of Tyr-190 is oriented perpendicular to the neighboring rings of Phe-190 and His-235 (Fig. 4). This arrangement allows a favorable interaction of the partially positively charged rims of Phe-190 and His-235 with the π-electron cloud of the central Tyr-190.

**Position 209**—Residue 209 is located close to the N terminus of α-helix Aα3 in domain A of BLA (Fig. 2). In wild-type BLA, there is a shallow hydrophobic indentation around the Cα atom of Ala-209 (Fig. 5). The base of this indentation is composed of residues Tyr-203 and Phe-240, whereas the rather hydrophilic rim is formed by the side chains of Lys-213, Asp-243, and Lys-237 as well as by carbonyl oxygen atoms from residues 203 through 206. Phe-240 is located in the opposing α-helix Aα4, which, together with Aα3, points toward the Ca-Na-Ca metal triad. There are five ordered water molecules covering the indentation.

As previously observed (18), the introduction of small hydrophobic residues in place of Ala-209 increases the half-life for the thermal inactivation of BLA by a factor of up to three. In the absence of structural information, it was argued that a favorable residue should contribute to the hydrophobic packing at the bottom of the indentation and should not disturb the pronounced water structure at the top (15). For the mutant A209V, the first premise is true, but the water structure is considerably diminished as indicated by the missing central water molecule and significantly higher B factors for the rest of the water molecules.

**Positions 264 and 265**—Residues 264 and 265 have historically been treated as a pair in mutational studies of BLA. They are located on the surface of the interface between domains A and B (Fig. 2). In wild-type BLA, residue 264 is a glutamine whose side chain is able to form hydrogen bonds with the side chain of Glu-189 in domain B. A possible deamidation of Gln-264 at high temperatures could place the resulting negatively charged side chain in an electrostatically unfavorable environment because of its close proximity to Glu-189 and Asp-266 (Fig. 6). Yet, as for Asn-190, there is no experimental proof that the removal of the amide side chain at this position reduces BLA deamidation.

Residue 265 (asparagine in wild-type BLA) shields a hydrophobic region composed of the underlying Pro-287 and Tyr-290. The crystal structure of the BLA variant containing a mutation of asparagine to tyrosine at this position shows that the tyrosine side chain participates in a complex network of aromatic interactions with Tyr-290, Trp-263, and His-289 (Fig. 6).

**DISCUSSION**

**Surface Hydrophobicity and Protein Stability**—It is generally assumed that amino acid substitutions on the surface do not affect the stability of proteins by large amounts. This is because most surface residues are involved in only a few transient interactions. Yet, this general rule suffers exceptions as exemplified here and in other studies. Our engineering work on BLA (10) demonstrates that a few point mutations at the surface are sufficient to drastically increase or decrease the natural high resistance of this enzyme toward thermal inactivation. Likewise, in some cases, it was shown that as few as one or two solvent-exposed residues confer substantial thermostability to natural or artificially engineered proteins (28–30).

Four of the stabilizing mutations examined in the present study replace amino acids that are either small and hydrophobic, or hydrophilic with larger, hydrophobic residues, resulting in an increased hydrophobic surface area. Our results thus seem to contradict another general concept, namely that, because of the energetic cost of solvating non-polar side chains, the exposure of hydrophobic residues at the protein surface is unfavorable for overall protein stability. Indeed natural proteins usually attempt to bury hydrophobic residues in the interior while placing hydrophilic residues at the surface. In line with this observation, a reduction in the hydrophobic surface area has often been recognized as an important factor contributing to the enhancement of stability in thermophilic proteins compared with their mesophilic counterparts (4). A similarly good correlation between increasing stability and decreasing surface hydrophobicity has also been found in mutagenesis studies involving solvent-exposed sites (28, 31, 32). However, radically different conclusions have been reached from other studies on the effect of surface hydrophobicity on protein folding and stability. It can indeed be argued that the introduction of hydrophobic residues at the surface is not necessarily thermodynamically unfavorable, if the non-polar side chains are more partially buried in the folded state than in the unfolded state (33). In the cold shock protein CspB from B. subtilis, three
surface-exposed phenylalanines participate both in activity and stability, and their replacement by alanine is highly destabilizing (34). Similarly, aromatic side chains that form clusters or are embedded in hydrophobic pockets at the protein surface have been shown to be critical for the thermal stability of a thermophilic protease (29, 35) and xylanase (36). In all these cases, the energy penalty associated with the partial exposure of the non-polar side chains in the folded protein must be overcompensated by the positive effect resulting from their partial burial and the stabilizing interactions they establish upon folding.

In the following we discuss the nature of the interactions that may account for the beneficial impact of the surface hydrophobic residues we introduced in our 5-fold mutant BLA.

**Hydrophobic Packing in Surface Indentations**—A close packing of the atoms in the interior of a protein is considered to be essential for stability. Reducing the number and volume of internal cavities through improved hydrophobic packing is an established strategy to stabilize its core and the protein overall (37–40). In contrast, because of the general belief that surface hydrophobicity is destabilizing, increasing the hydrophobic packing within surface indentations has rarely been considered in mutagenesis studies. In the case of our hyperthermostable BLA variants, there are at least two mutations, A209V and H133V, for which the stabilizing effects are likely a result of increased hydrophobic packing of surface indentations. Both mutation sites lie in a small surface groove or cavity formed between secondary structure elements: between three strands of the large β-sheet of domain B (position 133) and between two helices of domain A (position 209). Hydrophobic contacts between these secondary structure elements are reinforced as a result of the mutations. In addition, substantial changes in the hydration shell of these indentations are likely to occur, as indicated by the exclusion of one ordered water molecule in the immediate vicinity of the mutation sites. The shielding from solvent of buried hydrophobic clusters and backbone structures may prevent the potential disruptive action of water molecules, as water molecules generally compete with main-chain atoms for hydrogen bonds and thereby disrupt secondary structures. The creation of a hydrophobic shield protecting β-sheet structures against invading water molecules has also been proposed for explaining the stabilizing effect of surface-located residues in the thermophilic protease and cold shock protein mentioned above (30, 35).

**Stabilization of β-Sheets**—At position 133, a good correlation was observed between the stabilizing effects of the 20 different amino acids and their β-sheet-forming propensity (18). As the first residue of an edge β-strand, residue 133 is expected to be key for the formation of the β-sheet structure within domain B. Analysis of our high resolution crystal structures shows that the dihedral angles at position 133 are more favorable for β-sheet formation in the mutant than in the wild-type structure. The structural changes are a consequence of reduced conformational stress around position 133 with the mutant side chain, thus allowing improved main-chain hydrogen bonding. The same conformational relaxation could be achieved by substitutions to other small amino acids, e.g. glycine or alanine. However, such substitutions do not increase the stability of BLA, supporting the idea that good β-sheet formers are preferred at this site. How the side chain of amino acids favor particular dihedral angles and thereby β-sheet formation, and to what extent the intrinsic β-sheet propensity is modulated by the local environment remains debatable (41–44). In the case of position 133 in BLA it is clear that the side chain determinants of β-sheet stability do not fully describe the effects of mutations; packing and solvation effects at the surface have to be taken into account as well.

**Aromatic-Aromatic Interactions**—The interaction between the side chains of aromatic residues can contribute about 1.3 to 6 kcal/mol to protein stability (45). Wild-type BLA naturally contains a large number of aromatic clusters at the surface, and their contribution to the overall stability of BLA is expected to be substantial. Note that hydrophobic residues at the surface are expected to be less thermodynamically unfavorable when clustered than when isolated since the energetic cost of solvating non-polar side chains is shared between the interacting residues. Interestingly, aromatic clusters are also observed at the surface of other glycoside hydrolases such as xylanase and cyclodextrin glucanotransferase (CGTase) from thermophilic organisms (36, 46). In starch-degrading enzymes, aromatic side chains serve as a stacking platform for the sugar moieties of the substrate. Their abundance at the surface may thus contribute to increased thermostability in the presence of starch, by allowing amylose chains to bind non-specifically to the protein (46).

In the hyperthermostable mutant amylase that is described here, two of the stabilizing substitutions (N190F and N265Y) introduce additional aromatic side chains at the protein surface. In both cases, the introduced side chain extends a network of aromatic-aromatic interactions of underlying residues to the outside of the protein. The aromatic ring of Tyr-265 is involved in a rather complex system of hydrophobic interactions, and its hydroxyl group is hydrogen bonded to water molecules. The aromatic interaction network involving Phe-190 is less complex, and its side chain is completely solvent-exposed. Despite those seemingly unfavorable characteristics, the N190F mutation is by far the most effective of all mutations characterized in BLA to date. The fact that leucine, but not tyrosine, is also beneficial at position 190 indicates that it is the pronounced hydrophobic character of the inserted residue that is the primary stability determinant at this surface site.

**The Importance of Metal Binding Sites**—Many mutations that influence the stability of BLA are located near the unique Ca-Na-Ca metal triad. The most stabilizing single site mutation observed in BLA so far is the N190F mutation. Phe-190 is located at the tip of a long loop of domain B that folds over domain A and forms part of the cage that entraps the metal ions. Although Phe-190 does not participate directly in metal binding, the triple aromatic interaction it forms together with Tyr-193 and His-235 acts as a clamp that may reduce the flexibility of the cage, thereby hindering the metal ions from diffusing out of the binding pocket at high temperatures where structural breathing is increased. The cage is composed of six aspartate residues that provide neutralizing negative charges for the metal ions. Once the metal ions diffuse out of their pockets, the high density of negative charges from the aspartates must severely destabilize this region. The N-terminal end of the nearby α-helices Aα3 and Aα4 point toward the metal-binding cage. Their helix dipoles therefore contribute partial positive charges for stabilizing the negatively charged aspartate residues in the metal-binding cage. In this respect, the mutation A209V, in addition to its groove-filling effect discussed above, also contributes to the stability of the triadic metal array as it lies in the middle of α-helix Aα3, therefore likely increasing the overall stability of the helix.

How sensitive the triadic metal binding array is with respect to the overall stability of BLA is demonstrated by the thermosensitive mutant N192A. Asn-192 stabilizes the metal binding cage by forming two hydrogen bonds between its polar side chain atoms and the main chain atoms of two direct metal ligands (Fig. 4). Removal of these hydrogen bonds leads to a...
BLA variant that can be expressed, but that is instantaneously inactivated when subjected to higher temperatures (19).

These results point to the central ternary metal binding site in BLA as one of the major nucleation sites for unfolding. Because of the high charge density in the ternary Ca-Na-Ca binding site, small changes in the stability of the metal binding cage are expected to lead to sharply cooperative, amplified effects on the overall stability of BLA. Furthermore, a cis-peptide bond between Trp-184 and Glu-185 is vital for maintaining the integrity of the metal binding cage. It is likely that this cis-peptide bond isomerizes at high temperatures once the metal ions have been removed from their binding pocket. Since the reverse process, the formation of non-proline cis-peptide bonds, is energetically extremely unfavorable (47), this isomerization could be the cause of the irreversibility of BLA unfolding.

In good agreement with this model, a two-stage unfolding transition has recently been proposed for the thermal inactivation of \textit{B. amyloliquefaciens} \(\alpha\)-amylase in which metal ion dissociation occurs in the first step immediately followed by irreversible unfolding (48). The loss of stabilizing bivalent ions has also been invoked as the first step of the unfolding reaction for other metallo-enzymes such as subtilisin (49) and xylose isomerase (50).

The detailed analysis of the structural aspects of metal binding in BLA as a result of various mutations is expected to be generally valuable for our understanding of the correlation between metal binding and protein stability. The introduction of metal binding sites has long been considered a promising general strategy to increase the stability of a protein (51). However, because of the structural complexity of metal binding sites, with contributions coming from sequentially separated regions, \textit{de novo} engineering is a formidable task. On the other hand, favorably modifying existing metal binding sites in metallo-proteins appears to be a feasible challenge (52).

\textbf{Kinetic Stability and the Theory of Helix-Coil Transitions—}

Examination of wild-type and mutant crystal structures at high resolution permits evaluation of the relative contribution of attractive forces to the overall thermodynamic stability of a protein. Using this approach, we partly explained the stabilizing effect of the seemingly unfavorable hydrophobic substitutions we introduced at the surface of BLA. Yet, the compensating local effects discussed above do not seem to fully account for the remarkable stabilizing effects observed with some of our BLA mutations, particularly N190F. Similarly, the dramatic destabilizing effect of the N192A mutation cannot be merely due to the energetic loss associated with the removal of two hydrogen bonds.

Protein folding and unfolding following first-order kinetics are highly cooperative processes that can be described by the theory of helix-coil transitions (53). In this context, “helix” denotes the ordered state of any kind of cooperatively formed structure, and “coil” denotes its disordered state. Helix-coil transitions can be separated into two steps, nucleation and propagation. In a protein, any region that tends to unravel upon external influences can act as a nucleation site for unfolding. However, because changes in the environment have their greatest impact on the surface of a protein, unfolding is expected to start at solvent accessible regions. There is a large energy barrier associated with nucleation, which renders this step the energetically most expensive part in a helix-coil transition. Once this barrier is overcome, less energy is required to drive the transition, which then progresses rapidly in a zipper-like fashion. Helix-coil transition theory accounts for the fact that relatively moderate changes in the local stability can lead to unpropotionally large overall effects, provided these changes occur at nucleation sites. We believe that the effects of mutations in BLA that lead to kinetically stabilized variants can be explained in this light. All mutated residues are involved in forming complex, cooperative substructures, namely metal binding, aromatic clusters, and regular secondary structural elements. They are exposed to bulk solvent and are located at the edge of these cooperatively formed substructures. These residues are therefore candidates for “weak points” where unfolding is initiated, e.g. through the effects of temperature. For the present structural work, we have focused on those mutations that have particularly strong positive influences on the kinetic stability of BLA. By doing so, we believe in retrospect that we have serendipitously selected for nucleation sites of BLA unfolding.

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