Thermostabilization by replacement of specific residues with lysine in a Bacillus alkaline cellulase: building a structural model and implications of newly formed double intrahelical salt bridges

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An alkaline, mesophilic endo-1,4-β-glucanase from alkaliphilic Bacillus sp. strain KSM-64 was significantly thermostabilized by replacement of both Asn179 and Asp194 with lysine by site-directed mutagenesis. Structural remodeling of the mutant enzyme newly generated by the double mutation suggested that Glu175→Lys179 and Glu190→Lys194 were the most plausible ion pairs, both of which involved side chains at the i and i+4 positions on the α-helix from Glu175 to Ser195. By molecular dynamics simulations, the Nδ hydrogens of Lys179 and Lys194 were found to coordinate with the carbonyl Oδ1 and Oδ2 of Glu175 and the carbonyl Oδ1 of Glu190, respectively, with distances of around 2 Å for all. These results confirm that the formation of these double intrahelical ion pairs (salt bridges) is responsible for the thermostabilization by the double mutation.

Keywords: Bacillus/Cellulase/intrahelix/ion pair/salt bridge/thermostability

Introduction

Alkaline endo-1,4-β-glucanases (Egls) (EC 3.2.1.4) from alkaliphilic Bacilli have been studied extensively for use as effective detergent additives (Hoshino and Ito, 1997; Ito, 1997; Horikoshi, 1999), including Egk-L for detergent powders (Yoshimatsu et al., 1990) and Egl-64 for liquid detergents (Shikata et al., 1990). A mutant Egl with higher thermostability is desirable because the most serious problem with detergent enzymes is thermal inactivation during isolation, granulation and storage. As is the case for detergent enzymes (Ito et al., 1998), one of the most pursued goals in protein engineering and biotechnology is the design of mutant proteins with increased thermostability. Many attempts have been made to relate the properties of enhanced thermostability to features inherent in the amino acid sequences and 3D structures of mesophilic and thermophilic enzymes (Vogt et al., 1997; Wallon et al., 1997; Závodszky et al., 1998; Deckerck et al., 2000). The enhancement of thermostability of certain proteins is brought about by increased internal hydrophobicity, increased polar surface area, shortening or deletion of surface loops, improved helix capping, additional ion pairs, increased occurrence of Pro residues, decreased helix-destabilizing residues and so on (e.g. Argos et al., 1979; Imanaka et al., 1986; Serrano and Fersht, 1989; Davies et al., 1993; Takano et al., 1995; Haney et al., 1997; Russell et al., 1997; Shirai et al., 1997b; Watanabe et al., 1997). It is very informative that a net increase in ionic interactions is often observed in thermophilic enzymes (Vogt et al., 1997; Wallon et al., 1997; Hashimoto et al., 1999; Kumar et al., 2000).

Recently, we found a thermostable, alkaline Egl (Egl-237) from the alkaliphilic Bacillus isolate KSM-S237 (Hakamada et al., 1997) and sequenced the cloned gene of the enzyme (Hakamada et al., 2000). The deduced amino acid sequence of Egl-237 showed high homology with those of Egk-K (Ozaki et al., 1990) and Egl-64 (Sumitomo et al., 1992). Based on the difference of a few amino acids, we constructed several chimeric genes from Egl-64 and Egl-237, and Lys179 and Lys194 residues in Egl-237, not conserved in Egl-64, were found to contribute to thermostability after site-directed mutagenesis. As a result, the double mutation Asn179→Lys (NK)/Asp194→Lys (DK) (NK/DK) significantly improved thermostability, the level of which was as high as that of the wild-type Egl-237 (Hakamada et al., 2001). One of the most striking features of thermostable enzymes, when compared with mesophilic enzymes, is the decrease in the number of Lys residues, which are mainly replaced with Arg and Glu residues (Argos et al., 1979; Davies et al., 1993; Shirai et al., 1997b). However, the thermal stabilization of mesophilic Egl-64 by the additional Lys residues instead of Arg residues is clearly contrary to these results with thermophilic enzymes. In spite of the gained basic Lys residue(s), the pI value of the mutant Egl-64 enzymes estimated by electrofocusing was slightly higher than or similar to that of the wild-type level. This suggests that the thermal stabilization achieved with the mutations involves the formation of new ion pairs, such as salt bridges, with some carboxylate residues localized on the enzyme surfaces. Although the 3D structures of glycosyl hydrase family 5 cellulases have been determined (Ducros et al., 1995; Sakon et al., 1996; Davies et al., 1998), their amino acid sequences and catalytic properties were too different from those of Egl-64 and Egl-237 in the same family to analyze the roles of the thermostabilizing Lys residues. Recently, we succeeded in crystallizing (Shirai et al., 1997b) and determining the 3D structure of the truncated Egk-K, designated Egk-Kt (Ozaki et al., 1995), similar to the two alkaline Bacillus Egls. Here we show the precise involvement of the specific Lys residues in and the mechanisms for the thermal stabilization of Egl-64 by homology modeling and implicating by molecular dynamics (MD) simulation.

Materials and methods

Bacterial strains and enzyme production

The alkaliphilic Bacillus sp. strain KSM-64 of our stock cultures was used as the source of the gene encoding Egl-64 (Sumitomo et al., 1992). Constructed plasmids were used individually for transformation of B. subtilis ISW1214 to exoproduce wild-type, NK, DK or NK/DK mutant enzyme in the medium described previously (Sumitomo et al., 1995; Hakamada et al., 2001).
Fig. 1. Effect of temperature on the stability of wild-type Egl-64 and its mutant enzymes. Each enzyme had previously been incubated for 30 min at the indicated temperatures in 0.1 M glycine–NaOH buffer (pH 9.0). The residual activities were measured under the standard conditions of enzyme assay. The original activity of each enzyme was taken as 100%.

- Wild-type Egl-64: ◆
- NK mutant: ○
- DK mutant: □
- NK/DK mutant: ■

Fig. 2. Structure-based amino acid sequence alignment of Egl-Kt and Egl-64. Sequences shown and residue numbers correspond to the mature proteins of Egl-K and Egl-64. The N- and C-termini of Egl-Kt correspond to Ala228 and Leu584, respectively, of mature Egl-K (Ozaki et al., 1995).

Amino acids are shown as the single-letter codes and asterisks above the sequences indicate the conserved residues. Open circles indicate the amino acid residues of Egl-64, replaced with lysine. Solid circles show the catalytic Glu residues (Hakamada et al., 2000). Underlines denote α-helices and double-underlines represent β-strands.

**Purification and assay of enzymes**

The recombinant enzymes produced by *B. subtilis* were purified by chromatography on a DEAE-Toyopearl 650C column to homogeneity as judged by SDS–PAGE. Egl activity was measured at 40°C in 0.1 M glycine–NaOH buffer (pH 9.0) with 1.0% CMC as substrate by the dinitrosalicylic acid procedure as described (Hakamada et al., 2000).

**Homology modeling of tertiary structures of Egl**

The structural models of Egl-64 and its engineered protein (NK/DK) were constructed by the homology modeling method, based on the 3D structure of Egl-Kt (PDB code No. 1go1) and the deduced amino acid sequence of Egl-64 (Sumitomo et al., 1992). All data sets were processed on a Silicon Graphics Indigo2 workstation using the InsightII/Discover software package (Molecular Simulation). MD simulations were calculated in an AMBER force field, where a dielectric constant ε of 1 and the partial atomic charges computed at pH 7 were used. The MD simulations were performed in 4000 fs after linear heating from 0 to 330 K within 1000 fs and the MD trajectory was saved at 20 fs intervals.

**Gene accession numbers**

The original nucleotide sequence data of Egl-64 have been deposited in the DDBJ, EMBL and GenBank databases under the accession No. M84963.

**Results and discussion**

**Cumulative thermostabilization by mutations NK and DK**

The thermostability of the NK, DK and NK/DK mutant enzymes was examined by heating each at various temperatures for 30 min in 0.1 M glycine–NaOH buffer (pH 9.0). As shown...
Thermostabilization of cellulase by intrahelical salt bridges

in Figure 1, the replacement of Asp194 with lysine (DK) thermostabilized Egl-64 significantly at high temperatures, as described (Hakamada et al., 2001). The stabilizing effect of the double mutation NK/DK was reproducibly cumulative, although the single NK mutation improved the thermostability only marginally. At 70°C, the half-lives ($t_{1/2}$) due to thermal inactivation increased in the order wild-type Egl-64 (9 min), NK mutant (15 min), DK mutant (38 min) and NK/DK mutant (41 min).

Structure-based alignment of amino acid sequences of Egl-64 and Egl-Kt

To allow accurate homology modeling and to understand better the mutagenesis results, the amino acid sequence of Egl-64 was aligned with that of Egl-Kt, as shown in Figure 2. High homology was observed within the suitably aligned sequences of both enzymes (73.6% identity and 94.4% similarity). The secondary structural elements, $\alpha$-helices and $\beta$-strands around the $\alpha_4$-helix region are colored red and cyan, respectively. The catalytic Glu residues are shown in the CPK representation.

Generation of intrahelical salt bridges by double mutation

In the modeled structure of the NK/DK mutant enzyme, the replacements of Lys residues at positions 179 and 194 form ion pairs with the side chains of Glu175 and Glu190, respectively, yielding double intrahelical ion pairs, as shown in Figure 4. Lys194 appears to interact also with Asp224 occurring on a $\alpha_4$-helix from Glu175 to Ser195 in the mutant Egl-64.

To examine the difference in the ionic interactions, the ion pair-forming Lys residues were MD simulated. Glu175, Glu176, Glu183 and Asp219 surrounding Lys194 were included in the simulations. In the results, the $N^i$ hydrogens on Lys179 interacted with the carbonyl $O^i$ and $O^{i+2}$ on Glu175, as shown in Figure 5A. In the MD trajectory, a minimum potential energy was obtained after 5000 fs (5000th step). In the case of the $N^i$ hydrogen on Lys194, the distance to the carbonyl $O^{i+1}$ on Glu190 was around 2 Å, whereas the distance to the carbonyl $O^{i+2}$ on Asp224 was small but frequently >3 Å (Figure 5B). Although the computed distances are shorter than expected, these results indicate that the most plausible ion pairs between Lys179 and Glu175 and between Lys194 and Glu190 is generated by the NK/DK mutation. Each pair involves side chains at the $i$ and $i + 4$ positions of the single $\alpha_4$-helix from Glu175 to Ser195 in the mutant Egl-64.

Marqusee and Baldwin (1987) showed that peptides containing $(i + 4)$ Glu–Lys forms more stable helices than peptides containing $(i + 3)$ Glu–Lys at extremes of pH (2 and 12) and also at pH 7. Hence we propose that the improved thermostability of the double mutation is due to the double intrahelical ion pairs (salt bridges). Moreover, the ion pair, the

Fig. 5. Deviation of distances (A) between Lys179 ($N^i$) and Glu175 ($O^i$ and $O^{i+2}$) in NK mutant and (B) between Lys194 ($N^i$) and Glu190 ($O^i$ and $O^{i+2}$) or Asp224 ($O^{i+1}$ and $O^{i+2}$) in DK mutant as a function of time. The simulations were conducted in 4000 fs at 330 K (4000 steps) after linear heating from 0 to 330 K within 1000 fs (1000 steps). Each simulation was repeated five times and typical results are shown. Identifications of the lines used for the course of the deviation of distance between each ion pair are indicated.

Fig. 6. Model structure of Egl-64 showing the catalytic residues and the salt bridges generated by Lys substitutions. (A) Overall model structure of the NK/DK mutant enzyme; (B) close-up view showing the region around the intrahelical double salt bridges. The solid ribbon diagrams were drawn using the program WebLab Viewer and $\alpha$-helices and $\beta$-strands around the $\alpha_4$-helix region are colored red and cyan, respectively. The catalytic Glu residues are shown in the CPK representation.
N² Lys194 hydrogen and the carbonyl Asp224 Oδ² may also be involved in the thermostabilization.

The Lys residues gained at positions 179 and 194 are exposed to the hydrophilic enzyme surfaces (Hakamada et al., 2001). Generally, solvent-exposed salt bridges appear to play little role in the stabilization of proteins as observed in the cases of T4 lysozyme (Dao-pin et al., 1991) and barnase (Serrano et al., 1990; Sali et al., 1991). In fact, individual salt bridges often do not contribute significantly to protein stability (Dao-pin et al., 1991; Hendsch and Tidor, 1994; Wimley et al., 1996). However, the cumulative or cooperative effects of a number of salt bridges or ion pairs may contribute substantially to stability enhancement in thermophilic proteins (Chan et al., 1995; Hennig et al., 1995; Yip et al., 1995; Vetriani et al., 1998; Hashimoto et al., 1999) and synthetic α-helical peptides (Kohn et al., 1997). The salt bridge Lys194–Glu175 in the less stabilized NK mutant is located on the N-terminus of the α4-helix immediately after a long random coil from Ala158 to Asn174 (see Figure 2). Therefore, the contribution of this ionic interaction may be limited and small owing to the mobility around this region. In contrast, the salt bridge Lys194–

References


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