39. **A General Principle of Increasing Protein Thermostability**

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The consensus on thermal stabilization of proteins has been achieved in two points. (1) Even one amino acid substitution on the polypeptide chain of a protein can enhance protein thermostability, although its effect is quite small in general.1) (2) The individual thermal stabilizations caused by such little structural changes are additive.2>-4) These imply that multiple amino acid substitutions on the polypeptide chain of a protein generate significant increases in protein thermostability. However, there is no principle which can predict both critical sites and residues in the sequence of a protein to be substituted for increasing protein thermostability.

We have found a strong correlation between the increase in number of proline residues and the rise in thermostability of five *Bacillus* oligo-1,6-glucosidases (dextrin 6-α-D-glucanohydrolase, EC 3.2.1.10) with very different thermostability.5)-8) On the basis of this finding, I wish to suggest a general principle of increasing protein thermostability.

Four oligo-1,6-glucosidases from a mesophile, *Bacillus cereus* ATCC 7064, a facultative thermophile, *Bacillus coagulans* ATCC 7050, a facultative thermophile able to grow at 30-66°C, *Bacillus thermoamylovuliquefaciens* KP 1071, and an obligate thermophile, *Bacillus thermoglucosidasius* KP 1006, are molecularly homologous monomeric globular proteins with a molecular weight (Mr) of about 60,000.5)-7)9) In contrast, oligo-1,6-glucosidase of an extreme thermophile, *Bacillus flavocaldarius* KP 1228, takes a homodimer structure, each subunit Mr being similar to those of the monomeric enzymes.8) The extreme thermophile enzyme exhibits a marked different amino acid composition. These five enzymes were compared in their thermostability (see Fig. 1, Tm ranges from 48°C to 91°C), amino acid composition and structural parameters derived from amino acid composition.7) From this analysis, several distinctive relationships have been discovered (Fig. 1). (i) Proline content increases in a linear fashion with the elevation of thermostability in the order, *B. cereus → B. coagulans → B. thermoamylovuliquefaciens → B. thermoglucosidasius → B. flavocaldarius* enzymes, but such a fine linearity fails to hold for all other residues. (ii) The structural parameters are quite similar among the enzymes. (iii) The potentials of salt bridge formation, hydrogen bonding and α-helix formation decrease slowly and straight against thermostability, but the potentials of β-sheet and β-turn formations remain nearly constant. (iv) The potential of hydrophobic interaction increases gradually as thermostability is enhanced, although the potential increase depends largely on the increase in proline content (cf. HP and HP' in Fig. 1).

Finding (ii) is indicative of the resemblance of these enzymes in the secondary and tertiary structures. Finding (iv) is compatible with evidence that
hydrophobic interactions unlike ionic interactions and hydrogen bondings are strengthened with increasing temperature.\(^\text{(10)}\) A proline residue in a polypeptide chain restricts severely the backbone conformational flexibility at both the proline itself and the preceding residue with a $\beta$-carbon.\(^\text{(11,12)}\) Proline residue shows a strong tendency to occur preferentially at the second site of $\beta$-turns.\(^\text{(13,14)}\) Findings (i–iv), in conjunction with these facts, suggest that the enhanced thermostability of *Bacillus* oligo-1,6-glucosidases is gained cumulatively by increasing the frequency of proline occurrence at the second sites of $\beta$-turns and the total hydrophobic residues, so that the folded polypeptide chain becomes more reduced in the backbone conformational entropy of unfolding as well as more improved in the hydrophobic interactions, resulting in the molecule being more tightened as a whole.\(^\text{(7)}\)

This proposal appeared to be given a strong support by Matthews *et al.*\(^\text{(12)}\) They could increase the stability of bacteriophage T4 lysozyme by replacing alanine with proline at one (position 82) of the $\beta$-turns so as to decrease the backbone entropy of unfolding. The observed effect was tiny, as the temperature of denaturation increased only by a degree of 0.8–2.1°C. This also seems to be the case for the oligo-1,6-glucosidases with a 1.2°C increase of denaturation due to the presence of an additional proline residue.\(^\text{(7)}\)

Very recently, we have determined the nucleotide sequences of three oligo-1,6-glucosidase genes from *B. cereus*, *B. coagulans* and *B. thermoglucosidasius*.\(^\text{(15,16)}\) The comparison of the amino acid sequences deduced from the nucleotide sequences demonstrates that
Proline residues occur at the \(\beta\)-turns with increasing frequency in parallel with increasing thermostability of the three enzymes.\(^{10}\)

The above examination naturally suggests a general principle of increasing protein thermostability, as follows. The thermostability of a globular protein (or an enzyme) can be enhanced cumulatively to a great extent by increasing the frequency of proline occurrence at the second sites of \(\beta\)-turns without significant alteration in the secondary and tertiary structures as well as in the function, whereby the backbone conformational entropy of unfolding is decreased successively. This overall effect will be further more heightened, when the side chain of proline residue can interact with the adjacent hydrophobic cavity.\(^{7}\) The increase in the frequency of proline occurrence at the second sites of \(\beta\)-turns is limited, as about one third of all amino acid residues in globular proteins are in \(\beta\)-turns and thus one twelfth in these second sites.\(^{17}\) \(B.\) flavocaldarius oligo-1,6-glucosidase gives the maximum frequency (8.52 mol% proline).\(^{8}\) It is very interesting how small the frequency is in psychrophilic \(Bacillus\) oligo-1,6-glucosidases. The principle is considerably useful to protein engineering, since it can specify straightforwardly the potential sites and residues (Xaa \(\rightarrow\) Pro) of substitution in the protein sequence to improve thermal stability.

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References