The conformational stability and folding mechanism of O^6-methylguanine-DNA methyltransferase from hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1

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Understanding the basis of protein stability and the folding mechanism is a problem of fundamental chemical and physical significance, and such knowledge is essential for numerous industrial applications. Hyperthermophilic organisms offer good model proteins for investigating the basis of protein stability and the folding mechanism because hyperthermophilic proteins exhibit high thermal and thermodynamic stability. This study investigates the basis of protein stability and the folding mechanism of O^6-methylguanine-DNA methyltransferase from hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 (*Tk*-MGMT).

Comparison of the conformational stability between *Tk*-MGMT and its mesophilic counterpart, AdaC, was performed in order to understand the equilibrium and kinetic properties of *Tk*-MGMT. *Tk*-MGMT unfolded at T_m = 98.6˚C, which was 54.8˚C higher than that of AdaC. The maximum free-energy changes (ΔG_{max}) were 2.6 times higher than those of AdaC. In addition, the temperature of ΔG_{max} of *Tk*-MGMT shifted considerably, by 22.1˚C, to a high temperature. The apparent heat capacity of the denaturation (ΔC_p) of *Tk*-MGMT was 0.7 times lower than that of AdaC. The data of ΔG-dependence on temperature indicate that *Tk*-MGMT acquires extreme thermal stability by the shifts of free energy versus temperature function towards higher temperatures and higher stabilities, and the flattening of this curves.

Four mutants were constructed by disrupting the intra- and inter-helical ion pairs in *Tk*-MGMT, and their stability were thermodynamically and kinetically investigated. One of the four mutants (E93A) was destabilized. ΔG of E93A was lower by ~ 4 kJ mol^{-1} than that of the wild type, and E93A unfolded one order of magnitude faster than the wild type and other variants. This indicates that the inter-helical and inter-domain ion pair network in the protein interior contributes to the stability of *Tk*-MGMT.

The kinetic stability of *Tk*-MGMT was also investigated as distinct from equilibrium stability. The half time for the unfolding of *Tk*-MGMT was 4.5 x 10^6 sec, which was two-order or more slower than those of AdaC and other mesophilic proteins. Furthermore, *Tk*-MGMT was tolerant to various chemical denaturants, such as guanidine hydrochloride, methanol, ethanol, 2-propanol, trifluoroethanol, and sodium dodecyl sulfate. Comparative study between the wild type and E93A shows that *Tk*-MGMT stabilizes under kinetic control even in irreversible unfolding conditions. It is found that *Tk*-MGMT showed slow unfolding kinetics as well as high equilibrium stability, leading to stabilizing the conformation of *Tk*-MGMT for a long time, even in denaturing conditions, and preventing aggregation.

Tk-MGMT shows slow unfolding kinetics, and therefore it is possible to investigate the unfolding mechanism in detail. Thus, the unfolding mechanism of *Tk*-MGMT was investigated by measuring far-UV CD and tryptophan fluorescence. *Tk*-MGMT was shown to unfold non-cooperatively via multiple unfolding steps. *Tk*-MGMT contained two different conformations in the physiological conditions, and they unfolded via different unfolding pathways. In addition, the intermediates that lose tertiary contact with α-helical conformation accumulated on both unfolding pathways. Unfolding intermediates have been found only rarely in earlier studies so far, and therefore how a protein unfolds is especially poorly understood. I was able to analyze the unfolding mechanism of *Tk*-MGMT in detail due to the slow unfolding kinetics of *Tk*-MGMT.

The information obtained from this study such as equilibrium stability and unfolding kinetics clarifies the mechanism of protein stability and enables us to prove enzyme stability and activity, leading to the useful application of enzymes in industry. Furthermore, the information on the unfolding pathways and intermediates provide the strong clues to understand the mechanism of protein unfolding, including protein aggregation and unfolding kinetics.