Comparative analyses of the conformational stability of a hyperthermophilic protein and its mesophilic counterpart

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Comparison of the conformational stability of an O6-methylguanine-DNA methyltransferase (MGMT) from the hyperthermophilic archaeon Thermococcus kodakaraensis strain KOD1 (Tk-MGMT), and its mesophilic counterpart C-terminal Ada protein from Escherichia coli (Ec-AdaC) was performed in order to obtain information about the relationship between thermal stability and other factors, such as thermodynamic parameters, thermodynamic stability and other unfolding conditions. Tk-MGMT unfolded at $T_m = 98.6 \, ^\circ C$, which was 54.8 $^\circ C$ higher than the unfolding temperature of Ec-AdaC. The maximum free energy ($\Delta G_{\text{max}}$) of the proteins were different; the value of Tk-MGMT (42.9 kJ·mol$^{-1}$ at 29.5 $^\circ C$) was 2.6 times higher than that of Ec-AdaC (16.6 kJ·mol$^{-1}$ at 7.4 $^\circ C$). The high conformational stability of Tk-MGMT was attributed to a 1.6-fold higher enthalpy value than that of Ec-AdaC. In addition, the $\Delta G_{\text{max}}$ temperature of Tk-MGMT was considerably higher (by 22.1 $^\circ C$). The apparent heat capacity of denaturation ($\Delta C_p$) of Tk-MGMT was 0.7-fold lower than that of Ec-AdaC. These three synergistic effects, increasing $\Delta G_{\text{max}}$, shifted $\Delta G$ vs. temperature curve, and low $\Delta C_p$, give Tk-MGMT its thermal stability. Unfolding profiles of the two proteins, tested with four alcohols and three denaturants, showed that Tk-MGMT possessed higher stability than Ec-AdaC in all conditions studied. These results indicate that the high stability of Tk-MGMT gives resistance to chemical unfolding, in addition to thermal unfolding.

Keywords: hyperthermostable protein, thermodynamics, thermal stability, archaea, O6-methylguanine-DNA methyltransferase.

Theoretical models were proposed to elucidate the mechanisms of thermostability. In the model A, high conversional stability leads to high temperature stability. In model B, low heat capacity change accompanying protein unfolding ($\Delta C_p$) is a result of the low dependence of $\Delta G$ on temperature; the protein therefore retains high stability at high temperature. In model C, the $\Delta G$ vs. temperature curve shifts to a higher temperature (see later). In model A, the high $T_m$ value of a protein is generally caused by high conformational stability. The activities of ribonuclease H from Thermus thermophilus and cellulase from Thermomonospora fusca are well explained by this model. In the flattened model B, the free energy profile of thermostable protein is shown as a flattened version of the mesophilic counterpart, $T_m$ increases with decreasing $\Delta C_p$ for unfolding of the protein. The activity of phosphorylcerate kinase from T. thermophilus is explained by this model, compared with its mesophilic counterpart from yeast [10]. The flattened model B is supported by thermodynamic analyses of other thermostable proteins, such as Sac7d from Sulfolobus acidocaldarius [12] and DNA-binding protein.
Sso7d from *Sulfolobus solfataricus* [13]. In the shifted model, C. *T*<sub>m</sub> increases directly with shifting of the Δ*G* vs. temperature curve to a higher temperature. Properties of histone proteins from *Methanothermobacter jannaschii* and *Pyrococcus* strain GB-3a can be explained by this model, compared with their mesophilic counterparts from *Methanobacterium formicicum* [14]. Although several hypotheses have been proposed for the increased stability of thermostable proteins when compared to mesophilic proteins, direct and systematic comparison between two such counterparts will be important in determining which hypothesis is correct.

In this paper, we examined thermodynamic parameters, in addition to various chemical unfolding conditions, of a hyperthermophilic *O*<sub>6</sub>-methylguanine-DNA methyltransferase (MGMT) from the hyperthermophilic archaean *Thermococcus kodakaraensis* strain KOD1 (Tk-MGMT, EC 2.1.1.63) and the data was compared to that of its mesophilic counterpart, the C-terminal domain (Met175−Arg354) of the Ada protein from *E. coli* (Ec-AdaC). In *E. coli*, cells, both the entire Ada protein and C-terminal fragments of Ada exist, and both are biologically functional [15]. KOD1 is one of the most thermostable organisms with an optimum growth temperature of 95 °C [16]. In high-temperature environments, proteins from KOD1 (including Tk-MGMT) must possess thermally adapted strategies based on amino-acid sequence, secondary structural alignment and/or three-dimensional structure. Tk-MGMT has a monomeric form with a molecular mass of 19.5 kDa and 174 amino acids [17], and its high resolution X-ray crystal structure has been solved previously [18]. The X-ray crystal structure of Ec-AdaC has shown that it has a monomeric form with a molecular mass of 19.6 kDa and 178 amino acids [19]. Although the amino-acid identity between the two proteins is rather low (∼20%), the three-dimensional structures of both proteins are similar. We have investigated the thermodynamic parameters, and discussed the global configuration of thermally adapted proteins. In addition, unfolding properties in several alcohols and denaturants were examined and the relationship between thermal stability and alcohol-tolerance was investigated.

**MATERIALS AND METHODS**

**Purification of Tk-MGMT**

Recombinant Tk-MGMT was expressed following the procedures described previously [16]. Protein concentrations were determined using a molar absorption coefficient of 18 000 m<sup>−1</sup>cm<sup>−1</sup> at 280 nm [20].

**Expression of Ec-adaC gene**

The *Ec-adaC* gene from *E. coli* was amplified by PCR. The primers of *Ec-adaC*-5′ (5′-CGAACCGTCTGATGACGCCTAAACAAAT-3′) and *Ec-adaC*-3′ (5′-GCTGCAGGATCCACATA-3′) were used as the amplification primers of C-terminal domain (Met175−Arg354) of Ec-Ada. Underlined sequences are the introduced restriction sites, NdeI and BamHI, respectively, and ligated to each other. The Ec-adaC gene was then cloned into pET-25b(+) and the vector was confirmed by DNA sequencing.

**Purification of Ec-AdaC protein**

Recombinant *Ec-AdaC* protein was overexpressed in *E. coli* BL21 (DE3) cells containing recombinant plasmid at 37 °C. When the *D<sub>660</sub>* reached 0.4, the expression was induced with 1 mM isopropyl thio-β-D-galactoside for 4 h. The cells from culture broth (2.5 L) were harvested by centrifugation and disrupted by sonication in 150 mL of 100 mM Tris/HCl buffer (pH 8.5) containing 1 mM EDTA. The supernatant was recovered after centrifugation at 15 000 g for 30 min at 4 °C. The supernatant was dialyzed overnight against 50 mM Tris/HCl buffer (pH 8.5) containing 1 mM EDTA and 0.1 mM dithiothreitol. The dialyzed sample was applied to a DEAE−Toyopearl column (5 cm × 15 cm; Tosho, Tokyo, Japan) equilibrated with 50 mM Tris/HCl buffer (pH 8.5) containing 1 mM EDTA. The adsorbed fraction was eluted with 50 mM Tris/HCl buffer (pH 8.5) containing 80 mM NaCl and 1 mM EDTA. The fraction was dialyzed overnight against 20 mM phosphate buffer (pH 6.5). The sample was applied to a HiTrap SP column (Pharmacia Biotech, Uppsala, Sweden) equipped with AKTA fast protein liquid chromatography system at 4 °C equilibrated with 20 mM phosphate buffer (pH 6.5). The sample was eluted with a linear gradient of 0−0.6 M NaCl containing 20 mM phosphate buffer (pH 6.5) at a flow rate of 0.8 mL·min<sup>−1</sup>. The fractions were collected and dialyzed overnight against 50 mM Tris/HCl buffer (pH 8.5). The sample was then applied to a Mono Q column (Pharmacia Biotech) equipped with the AKTA fast protein liquid chromatography system at 4 °C equilibrated with 50 mM Tris/HCl buffer (pH 8.5). The sample was eluted with a linear gradient of 0−0.2 M NaCl containing the 50 mM Tris/HCl buffer (pH 8.5) at a flow rate of 0.8 mL·min<sup>−1</sup> and the eluted sample was stored at 4 °C. Homogeneity of the purified protein was confirmed by SDS/PAGE. The mass of the purified protein was confirmed by mass spectroscopy (LCQ-MS, Finnigan, Bremen, Germany). The measured mass of the protein was 19609.0 Da at pH 8.5, which was in good agreement with the mass of *Ec-AdaC* calculated from the amino-acid sequence (19610.3). The concentration of *Ec-AdaC* was calculated from absorbance at 280 nm using the molar absorption coefficient 15 900 m<sup>−1</sup>cm<sup>−1</sup> [20].

**CD spectra**

Far-UV CD spectra were measured with a Jasco spectropolarimeter, model J-720 W (Japan Spectroscopic Company, Tokyo, Japan) equipped with a thermal incubation system. The far-UV CD spectra of Tk-MGMT and Ec-AdaC were measured at a protein concentration of 0.1 mg·mL<sup>−1</sup> with a 2-mm cuvette.

**GdnHCl titration and stability curve data analysis**

GdnHCl induced unfolding profiles of Tk-MGMT and Ec-AdaC were measured by CD ellipticity at 222 nm. For the equilibrium measurement, 50 μL protein solution was mixed with 450 μL of 10 mM Tris/HCl buffer (pH 8.0)
containing various concentrations of GdnHCl. The samples were then incubated for three days at 37 °C (Tk-MGMT) or 20 °C (Ec-AdaC).

The experimental data were analyzed by a two-state folding mechanism. Briefly, the apparent equilibrium constant ($K_{app}$) during the unfolding process was determined according to equation,

$$K_{app} = \frac{f_U}{f_N} = \frac{y_N - y}{y - y_U}$$  \hspace{1cm} (1)

where $f_N$ and $f_U$ are the fractions of the folded and unfolded states, and $y_N$, $y_U$, and $y$ represent the signals of the folded, unfolded, and measured intensity, respectively. The conformational free energy change ($\Delta G$) of the unfolding process is related to the apparent equilibrium constant by $\Delta G = -RT\ln K_{app}$. A two-state unfolding model can assume a linear dependence between $\Delta G$ and the denaturant concentration as shown in equation,

$$\Delta G = \Delta G_{H_{2}O} - m[\text{denaturant}]$$  \hspace{1cm} (2)

where $m$ values represents the dependence of the $\Delta G$ on denaturant.

In order to obtain the temperature dependence of the free energy, the GdnHCl induced unfolding profiles of Tk-MGMT were measured at 10, 20, 30, 40, 50, 60 and 70 °C; those of Ec-AdaC were measured at 5, 10, 15, 20, 25, 30 and 35 °C. The free energy changes dependent on temperature were plotted in the figure. Here, $\Delta G$ is expressed as a function of the melting temperature ($T_m$), the change of enthalpy due to protein unfolding at $T_m$ ($\Delta H_m$), and the heat capacity change accompanying protein unfolding ($\Delta C_p$) according to Gibbs–Helmholtz equation.

$$\Delta G = \Delta H_m \left(1 - \frac{T}{T_m}\right) - \Delta C_p \left(T_m - T\right) + T \ln \left(\frac{T}{T_m}\right)$$  \hspace{1cm} (3)

Using Eqn. (3), a calculation program (igor pro 3.0, WaveMatrics Inc., Oregon, USA) was instructed to fit $T_m$, $\Delta H_m$, and $\Delta C_p$.

Unfolding titration with various chemical conditions

Unfolding titration of Tk-MGMT and Ec-AdaC against methanol (MeOH), ethanol (EtOH), isopropanol (PrOH), trifluoroethanol, urea, and SDS were monitored by far-UV CD spectra at 20 °C. Tk-MGMT or Ec-AdaC was dissolved with respective denaturant concentration [every 5% (v/v) for MeOH, EtOH and PrOH; every 0.01% (w/v) for SDS; every 0.2 M for urea], then incubated for 1 h at 20 °C. The far-UV spectra were measured at a protein concentration of 0.1 mg·mL$^{-1}$ in 50 mm Tris/HCl buffer (pH 8.0) or 50 mm citrate/HCl buffer (pH 3.0).

**RESULTS**

Reversibility of Tk-MGMT and Ec-AdaC

Figure 1 shows far-UV CD spectra of Tk-MGMT and Ec-AdaC. The far-UV CD spectrum of Tk-MGMT in the absence of GdnHCl showed minima at 208 nm and 222 nm; the spectrum showed a typical $\alpha$ helical shape (Fig. 1A). The $[\theta]$ at 222 nm was 10 500 deg·cm$^{-2}$·dmol$^{-1}$. In 7.2 M GdnHCl, the far-UV CD intensity decreased and exhibited a typical unfolded spectrum. The spectra at wavelengths shorter than 215 nm could not be monitored due to the high level of background noise. The fully unfolded Tk-MGMT with 7.2 M GdnHCl was dialyzed against the buffer and the far-UV CD spectrum was measured. The spectrum of dialyzed protein showed it was fully refolded. This was confirmed with the far-UV CD intensity. Figure 1B shows the far-UV CD spectra of Ec-AdaC. The intensity of the far-UV CD of Ec-AdaC was of similar magnitude to that of Tk-MGMT; the $[\theta]$ at 222 nm was 11 100 deg·cm$^{-2}$·dmol$^{-1}$. At the concentration of 2.4 M GdnHCl, the far-UV CD intensity decreased, showing typical unfolded spectrum. The fully unfolded Ec-AdaC with 2.4 M GdnHCl was dialyzed against the buffer and the far-UV CD spectrum was measured. These data showed that Tk-MGMT and Ec-AdaC are able to refold from GdnHCl induced unfolded states. The intensity of the ellipticity at 222 nm was used for the detection of unfolding profiles for both Tk-MGMT and Ec-AdaC.

Determination of thermodynamic parameters

Figure 2 shows GdnHCl induced unfolding profiles of Tk-MGMT and Ec-AdaC. The titration curve of Tk-MGMT was highly cooperative from 4 M to 6 M GdnHCl, while the unfolding of Ec-AdaC started at 0.6 M GdnHCl, indicating that the effect of denaturant on conformations of two proteins was considerably different. However, the $m$ values

![Fig. 1. Far-UV CD spectra of Tk-MGMT (A) and Ec-AdaC (B). Continuous lines show the spectra in 10 mm Tris/HCl (pH 8.0). Dashed lines show the spectra of Tk-MGMT in 7.2 M GdnHCl or Ec-AdaC in 2.4 M GdnHCl. Dotted lines show the spectra of the refolded Tk-MGMT and Ec-AdaC by dialysis.](image-url)
describing the GdnHCl concentration dependence of $\Delta G$ were similar between the two proteins. The $m$ values of $Tk$-MGMT and $Ec$-AdaC at 20 °C were 7.8 kJ·mol$^{-1}$·M$^{-1}$ and 8.0 kJ·mol$^{-1}$·M$^{-1}$, respectively. The conformational free energy was calculated using the $m$ values; the $\Delta G$ of $Tk$-MGMT and $Ec$-AdaC at 20 °C were 41.3 kJ·mol$^{-1}$ and 14.3 kJ·mol$^{-1}$, respectively.

GdnHCl induced unfolding curves at various temperatures were measured in order to determine the thermodynamic parameters, $\Delta G$, $m$ value and midpoint of the unfolding concentration of GdnHCl, at various temperatures (Tables 1 and 2). The unfolding titration curves of $Tk$-MGMT and $Ec$-AdaC were successfully monitored in a two-state manner at below 70 and 35 °C, respectively. The $\Delta G$ of $Tk$-MGMT and $Ec$-AdaC as a function of temperature were plotted using the values in Table 1 and Table 2 (Fig. 3). These two stability curves were fitted to the Gibbs–Helmholtz equation (Eqn. 3) and the thermodynamic parameters were determined (Table 3). The maximum free energies ($\Delta G_{\text{max}}$) of $Tk$-MGMT and $Ec$-AdaC were 42.9 kJ·mol$^{-1}$ at 29.5 °C and 16.6 kJ·mol$^{-1}$ at 7.4 °C, respectively. Thermodynamic stability of the two proteins was extremely different; the $\Delta G_{\text{max}}$ of $Tk$-MGMT was 2.6 times higher than that of $Ec$-AdaC. In addition, the difference in $T_{\text{m}}$ (the temperature at which $\Delta G$ is at a maximum) between the two was 22.1 °C. Because of synergistic effect of differences in $\Delta G_{\text{max}}$ and $T_{\text{m}}$, the transition midpoint for thermal unfolding ($T_{\text{m}}$) between $Tk$-MGMT (98.6 °C) and $Ec$-AdaC (43.8 °C) were also notably different ($\Delta T_{\text{m}} = 54.8 °C$).

In addition, the $\Delta C_p$ of $Tk$-MGMT (5.2 kJ·mol$^{-1}$·K$^{-1}$) was slightly low compared to that of $Ec$-AdaC (7.4 kJ·mol$^{-1}$·K$^{-1}$). Therefore, the shape of the $\Delta G$ curve of $Tk$-MGMT showed mixed profile of three kinds of features, high, shifted to high temperature, and slightly broad, compared to those of $Ec$-AdaC.

### Unfolding under various chemical conditions

Far-UV CD spectra of $Tk$-MGMT and $Ec$-AdaC with MtOH, EtOH, PrOH, trifluoroethanol, GdnHCl, urea and SDS were monitored. The concentrations of alcohols, urea, GdnHCl, and SDS were increased by 5% (v/v), 0.2 m, 0.2 m, and 0.01% (w/v). Figure 4 shows the typical far-UV CD spectra of $Tk$-MGMT. The far-UV CD spectra of

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$\Delta G$ (kJ·mol$^{-1}$)</th>
<th>$m$ (kJ·mol$^{-1}$·M$^{-1}$)</th>
<th>[GdnHCl]$_{\text{50%}}$ (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>37.9 ± 2.2</td>
<td>7.3 ± 0.4</td>
<td>5.14 ± 0.02</td>
</tr>
<tr>
<td>20</td>
<td>40.6 ± 2.5</td>
<td>7.8 ± 0.4</td>
<td>5.15 ± 0.02</td>
</tr>
<tr>
<td>30</td>
<td>41.9 ± 2.5</td>
<td>8.1 ± 0.4</td>
<td>5.15 ± 0.02</td>
</tr>
<tr>
<td>40</td>
<td>41.3 ± 2.7</td>
<td>8.0 ± 0.5</td>
<td>5.14 ± 0.02</td>
</tr>
<tr>
<td>50</td>
<td>32.5 ± 2.2</td>
<td>6.5 ± 0.4</td>
<td>4.98 ± 0.03</td>
</tr>
<tr>
<td>60</td>
<td>32.9 ± 1.9</td>
<td>7.2 ± 0.4</td>
<td>4.51 ± 0.02</td>
</tr>
<tr>
<td>70</td>
<td>25.7 ± 2.0</td>
<td>6.6 ± 0.4</td>
<td>3.88 ± 0.03</td>
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</table>

Table 2. $\Delta G$ and $m$ values of $Ec$-AdaC at various temperatures in 50 mTris/ HCl (pH 8.0).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$\Delta G$ (kJ·mol$^{-1}$)</th>
<th>$m$ (kJ·mol$^{-1}$·M$^{-1}$)</th>
<th>[GdnHCl]$_{\text{50%}}$ (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>15.9 ± 0.9</td>
<td>10.5 ± 0.6</td>
<td>1.52 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>15.2 ± 1.0</td>
<td>9.6 ± 0.6</td>
<td>1.58 ± 0.02</td>
</tr>
<tr>
<td>15</td>
<td>14.1 ± 1.1</td>
<td>8.8 ± 0.7</td>
<td>1.60 ± 0.02</td>
</tr>
<tr>
<td>20</td>
<td>12.4 ± 0.9</td>
<td>8.0 ± 0.6</td>
<td>1.56 ± 0.02</td>
</tr>
<tr>
<td>25</td>
<td>10.7 ± 0.8</td>
<td>7.2 ± 0.5</td>
<td>1.48 ± 0.02</td>
</tr>
<tr>
<td>30</td>
<td>8.4 ± 1.2</td>
<td>6.3 ± 0.8</td>
<td>1.32 ± 0.05</td>
</tr>
<tr>
<td>35</td>
<td>5.5 ± 1.8</td>
<td>5.4 ± 1.1</td>
<td>1.03 ± 0.13</td>
</tr>
</tbody>
</table>

![Fig. 3. Conformational free energies of $Tk$-MGMT and $Ec$-AdaC as a function of temperature. The free energy of $Tk$-MGMT (closed circles) and $Ec$-AdaC (open circles) were plotted. Each data set was fitted to the Gibbs–Helmholtz equation (Eqn 3) as shown in Materials and methods.](Image)
**Table 3. Thermodynamic parameters of Tk-MGMT and Ec-AdaC.** $T_{\text{m}}$ was the temperature at $\Delta G_{\text{m}}$. $\Delta G_{\text{m}}$ was the maximum value of $\Delta G$ obtained from $\Delta G$ vs. temperature dependent profile; the data was from experiments performed in 50 mM Tris/HCl (pH 8.0).

<table>
<thead>
<tr>
<th></th>
<th>$T_{\text{m}}$ (C)</th>
<th>$\Delta C_{\text{p}}$ (kJ mol$^{-1}$K$^{-1}$)</th>
<th>$\Delta H(T_{\text{m}})$ (kJ mol$^{-1}$)</th>
<th>$T_{\text{max}}$ (C)</th>
<th>$\Delta G_{\text{max}}$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tk-MGMT</td>
<td>98.6 ± 6.1</td>
<td>5.2 ± 1.4</td>
<td>419 ± 53</td>
<td>29.5</td>
<td>42.9</td>
</tr>
<tr>
<td>Ec-AdaC</td>
<td>43.8 ± 0.5</td>
<td>7.4 ± 0.5</td>
<td>284 ± 8</td>
<td>7.4</td>
<td>16.6</td>
</tr>
</tbody>
</table>

Tk-MGMT in 50% EtOH at pH 8.0 agreed with that of the native structure. With increasing concentration, the far-UV CD intensity decreased. In 60% EtOH, the sample was aggregated and the far-UV CD intensity clearly decreased. At pH 8.0, Tk-MGMT aggregated with increasing concentration of MtOH, EtOH, and PrOH. At high concentrations of trifluoroethanol at pH 8.0, the $\alpha$ helical content increased, showing typical alcohol-induced unfolded spectra [21]. Table 4 shows the rates of unfolded molecules in MtOH, EtOH, PrOH, and trifluoroethanol. The native CD spectra of Tk-MGMT were retained at concentrations of 60% MtOH, 50% EtOH, 45% PrOH, and 45% trifluoroethanol at pH 8.0. In acidic conditions at pH 3.0, aggregation was not observed with all alcohols. The order of effectiveness of alcohols to Tk-MGMT was shown to be trifluoroethanol > PrOH > EtOH > MtOH, showing the same order as previously reported for $\beta$ sheet protein of $\beta$-lactoglobulin and $\alpha$ helical protein of melittin [22]. Alcohol-induced unfolding of Ec-AdaC was examined and is summarized in Table 4. Ec-AdaC unfolded at lower concentrations than Tk-MGMT in all alcohols. The titration analyses for Tk-MGMT and Ec-AdaC in GdnHCl, urea, and SDS were performed (Table 4). The native CD spectra of Tk-MGMT were retained at concentrations of 4.7 M, > 8.0 M, and 0.10% in GdnHCl, urea, and SDS at pH 8.0, respectively. In particular, Tk-MGMT was not fully unfolded by urea at pH 8.0. In all chemical denaturants, Tk-MGMT was more stable than Ec-AdaC; the native CD spectra of Ec-AdaC was retained at concentrations of 0.8 M, 1.8 M, and 0.07% in GdnHCl, urea, and SDS at pH 8.0, respectively. At pH 3.0, GdnHCl-, urea-, and SDS-induced unfolding concentrations of Ec-AdaC were lower than those of Tk-MGMT. These data showed that Tk-MGMT was stable in various unfolding solutions when compared to Ec-AdaC, although these chemical conditions affect protein conformations in a number of different ways.

**Table 4. Concentration for retaining native structure in various conditions with 50 mM Tris/HCl (pH 8.0) or 50 mM Citrate-HCl (pH 3.0) observed by far-UV CD spectra of Tk-MGMT and Ec-AdaC.**

<table>
<thead>
<tr>
<th></th>
<th>Tk-MGMT</th>
<th>Ec-AdaC</th>
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<tbody>
<tr>
<td></td>
<td>pH 3.0</td>
<td>pH 8.0</td>
</tr>
<tr>
<td>MtOH (± 5%)</td>
<td>60 (60$^a$)</td>
<td>55 (30$^a$)</td>
</tr>
<tr>
<td>EtOH (± 5%)</td>
<td>45 (50$^a$)</td>
<td>45 (40$^a$)</td>
</tr>
<tr>
<td>PrOH (± 5%)</td>
<td>30 (45$^a$)</td>
<td>40 (35$^a$)</td>
</tr>
<tr>
<td>trifluoroethanol (± 5%)</td>
<td>25 (45)</td>
<td>25 (10$^a$)</td>
</tr>
<tr>
<td>GdnHCl (± 0.2 M)</td>
<td>3.2 (4.7)</td>
<td>0.6 (0.8)</td>
</tr>
<tr>
<td>Urea (± 0.2 M)</td>
<td>6.4 (&gt; 8.0)</td>
<td>1.4 (1.8)</td>
</tr>
<tr>
<td>SDS (± 0.01%)</td>
<td>0.12 (0.10)</td>
<td>0.06 (0.07)</td>
</tr>
</tbody>
</table>

$^a$ Aggregation was observed at and beyond the indicated concentration.

**DISCUSSION**

Hyperthermophiles grow optimally above 80 °C. Therefore, proteins from hyperthermophiles retain their native structure even at high temperatures. In this report we have studied the conformational stability of hyperthermophilic Tk-MGMT and compared its properties with its mesophilic counterpart, Ec-AdaC, to clarify the relationship between thermostability, several thermodynamic parameters and the chemical unfolding conditions.

Based on the theoretical thermodynamics, three different models, namely high stability model A, flattened model B, and shifted model C, have been proposed to explain the dependence of free energy of a thermostable protein [10]. A direct relationship between the thermal stability and thermodynamic stability is assumed in the high stability

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**Fig. 4. Typical far-UV CD spectra in various solutions.** (A) Far-UV CD spectra of Tk-MGMT in 0% (bold continuous line), 50% (thin continuous line), 55% (dotted line), 60% (broken line) EtOH at pH 8.0. (B) Far-UV CD spectra of Tk-MGMT native form (continuous line), with 50% trifluoroethanol (dotted line), and with 0.16% SDS (broken line) at pH 8.0.
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model A, and the model predicts that the overall free energy change ($\Delta G$) for unfolding of the protein at any temperature is higher than that of the mesophilic counterpart. Our experimental results have shown that the heat tolerance of Tk-MGMT cannot be explained by only one of these three strategies. Thermostability of Tk-MGMT can be understood by thermal adaptation with the combined strategies of model A, B, and C. The difference in $T_{\text{max}}$, the temperature at which $\Delta G$ is a maximum, between the two proteins was 22.1 °C, indicating the curve of $\Delta G$ vs. temperature was clearly shifted to a higher temperature. The $\Delta G_{\text{max}}$ of Tk-MGMT was 42.9 kJ·mol$^{-1}$, which is 2.6 times higher than that of Ec-AdaC. In addition, $\Delta C_p$ of Tk-MGMT was 0.7 times lower than that of Ec-AdaC. The thermal adapted strategy of the synergistic effects of Tk-MGMT is a novel property among hyperthermostable proteins.

In order to explain the reason why the profile of a $\Delta G$ vs. temperature curve of Tk-MGMT shifts to a higher temperature and increases in value, thermodynamic parameters were compared. The $\Delta C_p$ is believed to arise from change primarily from the exposure of hydrophobic residues during unfolding [23], suggesting that the $\Delta C_p$ depends on the size of proteins. For example, the values for thioredoxin (108 amino acids), barnase (110 amino acids), and ribonuclease A (124 amino acids) are 7.0, 5.9 and 5.9 kJ·mol$^{-1}$·K$^{-1}$, respectively [24–26]. The $\Delta C_p$ values of Tk-MGMT (174 amino acids, 5.2 kJ·mol$^{-1}$·K$^{-1}$) and Ec-AdaC (178 amino acids, 7.4 kJ·mol$^{-1}$·K$^{-1}$) were quite reasonable based on the sizes. However Tk-MGMT exhibited a slightly (0.7-fold) lower $\Delta C_p$ than Ec-AdaC, indicating that the curve of the $\Delta G$ vs. temperature of Tk-MGMT is flattened. The shape of the curve therefore results in the $T_m$ shifting to a higher temperature. Low $\Delta C_p$ might be accomplished by maintaining a cavity in the protein core in the native structure, or retaining hydrophobic regions in the unfolded structure. The hypothesis of the presence of a cavity is not supported by us, because our structural analysis is not in agreement with this hypothesis [18]. Hence, the latter theory that the structure is not fully unfolded by the thermal or chemical unfolding environments would seem to be correct.

The $\Delta G_{\text{max}}$ of Tk-MGMT was 2.6 times higher than that of Ec-AdaC, indicating that the protein was stabilized by the strategy of model A. Another possibility for thermal stability of Tk-MGMT was higher $\Delta G_{\text{max}}$, resulting from the high enthalpy change [$\Delta H(T_m)$].

The strategy for thermal adaptation of Tk-MGMT also agreed with model C. The shift of the $\Delta G$ vs. temperature curve to a higher temperature directly contributed to thermal stability. One of the possible reasons is that ion-pair networks on the protein surface affect the thermal stability, rather than conformational stability. It has been reported that ion-pairs on protein surface do not contribute to protein stabilization [27–30], but rather participates in the thermal stability [31,32]. Based on the structural analysis of the Tk-MGMT, unique ion-pair networks were observed in specific locations on helices [18]. Therefore they may be involved in protein stabilization.

Figure 5 summarizes mechanisms of thermostabilization. Curve 1 indicates a profile of typical mesophilic protein, such as Ec-AdaC. By increasing only $\Delta H$, curve 1 moves towards curve 2 (high stability model A). The higher $\Delta H$ corresponds to the favorable interactions between side-chains in the native structure. On the other hand, by increasing $T_m$, curve 1 moves toward curve 3 (shifted model C). This feature may be explained by increased number of surface ion-pair networks. In the case of Tk-MGMT, protein is stabilized by the combined effect of high stability model and shifted model as shown by curve 4.

$\Delta G$ values of Tk-MGMT and Ec-AdaC at optimum growth temperatures are almost identical ($\approx$ 10 kJ·mol$^{-1}$) even though the $\Delta G_{\text{max}}$ values are different (Fig. 3), possibly suggesting that proteins are generally optimized to acquire moderate thermodynamic stability at optimum temperatures for their functions.

A number of studies of alcohol-induced denaturation of proteins have previously been reported [21,22]. However, there is little data on the comparative analysis of alcohol-induced folding between hyperthermophilic and mesophilic proteins and a higher stability of the former towards alcohol or organic solvents has not been demonstrated. The studies here clearly show that Tk-MGMT is more resistant to such denaturation. It may well be that a reduction in the number of long loops and/or surface hydrophobic residues in hyperthermophilic proteins is important in this regard.

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**REFERENCES**


