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Equilibrium and Kinetic Stability of a Hyperthermophilic Protein, O^6-Methylguanine-DNA Methyltransferase under Various Extreme Conditions

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In this work we have studied the equilibrium and kinetic stability of a hyperthermophilic protein, O^6-methylguanine-DNA methyltransferase (*Tk*-MGMT), and its mesophilic counterpart AdaC, in various chemical solutions. In an unfolding experiment using guanidine hydrochloride (GdnHCl), the unfolding free-energy change of *Tk*-MGMT at 30°C was 42.0 kJ mol^{-1}, and the half time for unfolding was 4.5×10^6 s, which is much slower than that of AdaC and representative mesophilic proteins. In unfolding experiments using methanol, ethanol, 2-propanol, trifluoroethanol (TFE), and sodium dodecyl sulfate (SDS), *Tk*-MGMT retained its native structure at high concentrations, despite the fact that these chemical solutions affect protein conformations in a number of different ways. Kinetic studies using TFE and SDS indicate that the unfolding rates of *Tk*-MGMT in these solutions are slow as in GdnHCl. Further, the results of a mutational experiment suggest that an ion-pair network plays a key role in this slow unfolding. This slow rate of unfolding under extreme conditions is a significant property that distinguishes *Tk*-MGMT from mesophilic proteins.

Key words: equilibrium stability, hyperthermophilic protein, kinetic stability, protein unfolding.

Abbreviations: CD, circular dichroism; EtOH, ethanol; GdnHCl, guanidine hydrochloride; MtOH, methanol; PrOH, 2-propanol; SDS, sodium dodecyl sulfate; TFE, trifluoroethanol; *Tk*, *Thermococcus kodakaraensis*.

Proteins from hyperthermophiles must retain a folded and functional structure at high growth temperatures with the requisite stability (*1*). The study of proteins from hyperthermophiles can give insights into the basis of protein stability, as well as providing ready-made stable proteins. In over a decade of work, several research groups have revealed the principles that govern how hyperthermophilic proteins maintain their native structures, even at the boiling temperature of water. Structural comparisons between hyperthermophilic proteins and their mesophilic counterparts suggest that thermal stability is achieved by small but relevant changes at different locations in the structure involving electrostatic interactions and hydrogen bonds, as well as by packing and hydrophobic effects (*2*, *3*). A comparison of the thermodynamics of the reversible unfolding of proteins from hyperthermophiles and mesophiles provided an insight into stability that could not be obtained from direct structural investigations. For example, the temperature dependence of free-energy changes provides a mechanism for the greater temperature resistance described by the Gibbs-Helmholtz equation (*4*).

Most studies of equilibrium and kinetic stability have been performed using mainly urea or GdnHCl. Accordingly, our knowledge of the equilibrium and kinetic properties of proteins in other denaturing solutions, such as organic solvents or detergents, is relatively poor. Organic solvents and detergents are likely to unfold proteins in different ways, because the endpoints of the unfolded states are different; chemical denaturants such as urea and GdnHCl unfold proteins into a completely unstructured state (*5*), while alcohols stabilize the non-native, α-helical conformation (*6*) and detergents unfold proteins into largely ordered states (*7*). Understanding the mechanism of solvent resistance has an interesting potential for industrial applications, including peptide and ester synthesis, as well as providing insight into protein stability.

We have recently reported the results of thermodynamic analyses of the O^6-methylguanine-DNA methyltransferase derived from *Thermococcus kodakaraensis* (*Tk*-MGMT) and its mesophilic counterparts (*8–12*). These studies have shown that *Tk*-MGMT possesses a higher equilibrium stability than its mesophilic counterpart. However, the kinetic stability of *Tk*-MGMT has not yet been studied. The kinetic stability of a protein leads to the persistence of enzyme activity, even under denaturing conditions. Therefore, it is important to investigate kinetic stability as distinct from equilibrium stability. The purpose of the present study was to understand the origin of the enhanced stability of proteins from hyperthermophiles by studying the equilibrium and

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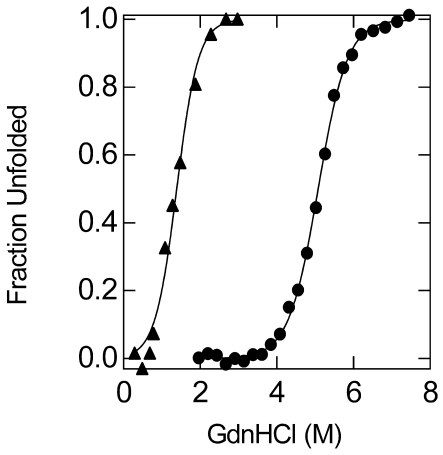

Fig. 1. **GdnHCl-induced titration curve of *Tk*-MGMT and AdaC monitored by CD at 222 nm at 30°C.** Solid lines represent the result of the nonlinear regression analysis according to Eq. 2. Closed circles, *Tk*-MGMT; closed triangles, AdaC.

kinetic parameters in various chemical solutions, including organic solvents and detergents, using *Tk*-MGMT as a model protein.

MATERIALS AND METHODS

Expression and Purification of Tk-MGMT and AdaC—Recombinant wild-type *Tk*-MGMT and mutant protein E93A were prepared as described previously (*13*). The protein concentrations were determined by absorbance at 280 nm using ε_{280} = 18,000 M^{-1} cm^{-1}.

AdaC was prepared as described previously (*12*). Protein concentrations were determined by absorbance at 280 nm using ε_{280} = 15,880 M^{-1} cm^{-1}.

CD spectra—Far-UV circular dichroism (CD) spectra were obtained on 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 were recorded at a protein concentration of 0.1 mg ml^{-1}.

Equilibrium Titration by GdnHCl—Tk-MGMT was incubated at 37°C for 1 day in the presence of 7.2 M GdnHCl for complete unfolding. The unfolded protein was mixed in 20 mM TrisHCl (pH 8.0) containing the desired concentration of GdnHCl. In order to reach complete equilibrium, the samples were incubated at 30°C for three days before CD measurement.

Equilibrium Titration with Alcohols and Detergent—Tk-MGMT and AdaC were mixed in 20 mM TrisHCl (pH8.0) containing the desired concentrations of ethanol (EtOH), 2-propanol (PrOH), trifluoroethanol (TFE), methanol (MtOH), or sodium dodecyl sulfate (SDS). After 30 min of incubation, CD signals at 222 nm were measured.

Unfolding Kinetics—The unfolding kinetics of *Tk*-MGMT was studied at various concentrations of GdnHCl, TFE, MtOH, and SDS in 20 mM TrisHCl (pH 8.0). Time-dependent unfolding was measured by CD 222 nm at 30°C. Manual mixing with a dead time of 1 s and continuous stirring were used throughout CD measurement.

Data Analyses—The equilibrium data were fitted by nonlinear least-squares analysis using the two-state model, based on the linear free energy relationship:

$$\Delta G = \Delta G^{H_2O} - m(\mathrm{GdnHCl}) \quad (1)$$

where ΔG is the free energy of unfolding, ΔG^{H_2O} is ΔG at 0 M GdnHCl, (GdnHCl) is the GdnHCl concentration, and m is the cooperative index of the transition. The observed signals (A_{obs}) at any concentration of GdnHCl are given by the following equation:

$$A_{obs} = \frac{A_N + A_U \exp\{-(\Delta G^{H_2O} - m[\mathrm{GdnHCl}/RT])\}}{1 + \exp\{-\Delta G^{H_2O} - m([\mathrm{GdnHCl}]/RT)\}} \quad (2)$$

where R and T are the gas constant and the absolute temperature, respectively. A_N and A_U are signals in the N and the U states, respectively, which are assumed to be linearly dependent on (GdnHCl).

In general, a plot of the logarithm of the rate constant for unfolding against the concentration of the denaturant is linear for many proteins at (GdnHCl) > $(GdnHCl)_{1/2}$ (*14*). The half time of unfolding ($t_{1/2}^{unf}$) of *Tk*-MGMT in the absence of a denaturant was calculated by fitting the data using least-squares analysis to the equation:

$$\log t_{1/2}^{unf} = \log t_{1/2}^{unf,H_2O} - m_u\,(\mathrm{GdnHCl}) \quad (3)$$

where $\log t_{1/2}^{unf}$ is the half time of unfolding, $\log t_{1/2}^{unf,H_2O}$ is $\log t_{1/2}^{unf}$ at 0 M GdnHCl, and m_u is the cooperative index of the transition.

Table 1. **Kinetic and thermodynamic parameters of representative small globular proteins.**

Protein	Structure	Denaturant	$(D)_{1/2}$[a] (M)	ΔG (kJ mol^{-1})	$t_{1/2}^{unf,H_2O}$ (s)	Temperature[b] (°C)	Reference
Cytochrome *c*	α	GdnHCl	2.9	28.8	4.1×10^{1}	23	(*27*)
CspB (*Bacillus subtilis*)	β-barrel	GdnHCl	1.5	11.3	7.0×10^{-2}	25	(*28*)
CspB (*Bacillus caldolyticus*)	β-barrel	GdnHCl	2.7	20.1	1.1	25	(*29*)
CspB (*Thermotoga maritima*)	β-barrel	GdnHCl	3.3	26.3	3.9×10^{1}	25	(*30*)
CI2	α/β	GdnHCl	3.9	29.3	3.9×10^{3}	25	(*31*)
Ubiquitin	α/β	GdnHCl	3.7	29.8	1.9×10^{3}	25	(*32*)
FKBP12	α/β	Urea	3.9	23.0	4.1×10^{3}	25	(*14*)
Muscle AcP	α/β	Urea	4.3	22.6	6.3×10^{3}	28	(*33*)
Spliceosomal protein U1A	α/β	GdnHCl	2.3	38.9	1.1×10^{4}	25	(*34*)
Tk-MGMT	α/β	GdnHCl	5.15 ± 0.05	42.0 ± 2.5	$4.5 \pm 2.4 \times 10^{6}$	30	
AdaC	α/β	GdnHCl	1.32 ± 0.05	8.4 ± 1.2	<1[c]	30	

[a]Midpoint of denaturant concentration. [b]Experimental temperature. [c]Unfolding was too fast for the precise value of $t_{1/2}^{unf,H_2O}$ to be determined.

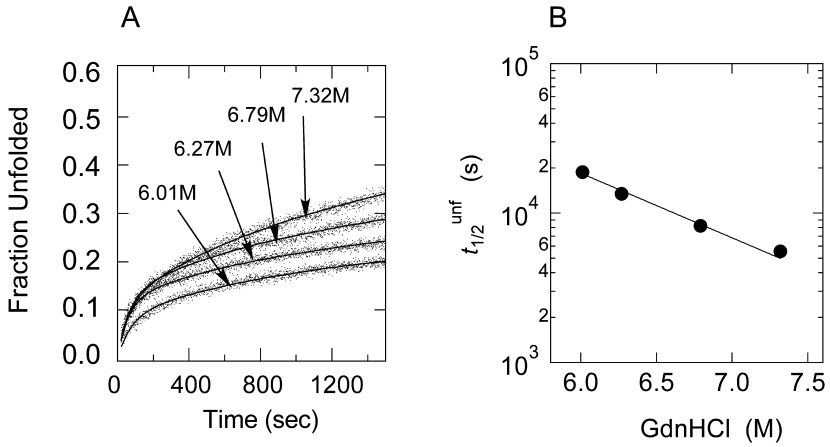

Fig. 2. **Kinetic traces of GdnHCl-induced unfolding monitored at CD 222 nm.** (A) Time course of unfolding of *Tk*-MGMT at 6.01, 6.27, 6.79, and 7.32 M GdnHCl. (B) The logarithm of $t_{1/2}^{unf}$ as a function of GdnHCl concentration. The $t_{1/2}^{unf,H_2O}$ values of *Tk*-MGMT were calculated according to Eq. 3. Solid lines represent the results of nonlinear regression analysis according to Eq. 3.

RESULTS AND DISCUSSION

Equilibrium Stability of Tk-MGMT—The equilibrium stability of *Tk*-MGMT was evaluated from a GdnHCl-induced titration curve, monitored by CD at 222 nm (Fig. 1). The values of ΔG and the midpoint of the denaturant concentration, $(GdnHCl)_{1/2}$, at 30°C were 42.0 ± 2.5 kJ mol^{-1} and 5.15 ± 0.05 M, respectively, assuming a two-state transition and a linear dependence of ΔG on the concentration of GdnHCl. The values of ΔG and $(GdnHCl)_{1/2}$ for AdaC at 30°C were 8.4 ± 1.2 kJ mol^{-1} and 1.32 ± 0.05 M, respectively. The data for AdaC were taken from reference (*9*). The ΔG and $(GdnHCl)_{1/2}$ values of *Tk*-MGMT are larger than those of AdaC and other mesophilic proteins reported previously (Table 1) (*15*). These results suggest that *Tk*-MGMT is stabilized by a relatively high free-energy change, which allows *Tk*-MGMT to retain its native structure even under conditions where the mesophilic protein has become denatured.

Unfolding Kinetics of Tk-MGMT—Figure 2A shows the progress of the GdnHCl-induced unfolding of *Tk*-MGMT as monitored by CD at 222 nm. The GdnHCl-induced unfolding of *Tk*-MGMT was initiated by diluting the native protein in 20 mM TrisHCl (pH 8.0) containing final GdnHCl concentrations of 7.32, 6.79, 6.27, and 6.01 M at 30°C. The $t_{1/2}^{unf}$ values for *Tk*-MGMT in 7.32, 6.79, 6.27, and 6.01 M GdnHCl were 5.5×10^3, 8.2×10^3, 1.3×10^4, and 1.8×10^4 s, respectively. GdnHCl-induced unfolding of AdaC was measured, but almost all of the reaction was completed within the dead time of 1 s. The $t_{1/2}^{unf}$ values of *Tk*-MGMT are apparently much smaller than those of AdaC. To ensure a more meaningful comparison among different proteins, the $t_{1/2}^{unf}$ values for *Tk*-MGMT in the absence of a denaturant ($t_{1/2}^{unf,H_2O}$) was calculated by extrapolating $t_{1/2}^{unf}$ back to zero-concentration denaturant as fitted to Eq. 3 (Fig. 2B). The estimated $t_{1/2}^{unf,H_2O}$ values for *Tk*-MGMT at 30°C is $4.5 \pm 2.4 \times 10^6$ s. Table 1 lists $t_{1/2}^{unf,H_2O}$ values for *Tk*-MGMT and published values for representative mesophilic proteins (*15*). The $t_{1/2}^{unf,H_2O}$ values for *Tk*-MGMT is at least two orders of magnitude higher than those of the mesophilic proteins. These results suggest that *Tk*-MGMT retains its native structure under extreme conditions due to its kinetic stability as well as its equilibrium stability.

Unfolding in Various Chemical Solutions—Figure 3 shows the far-UV CD spectra of *Tk*-MGMT in 20 mM TrisHCl (pH 8.0) containing MtOH, EtOH, PrOH, TFE, GdnHCl, and SDS. A variety of spectra were observed depending on the denaturing conditions. Typically, four types of spectra were observed. MtOH, EtOH, and PrOH denatured *Tk*-MGMT into aggregates, resulting in a decrease in far-UV CD intensity, with a minimum at 225 nm. TFE denatured *Tk*-MGMT into a typical alcohol-induced α-helical conformation. SDS denatured *Tk*-MGMT irreversibly with a far-UV CD spectrum similar to that of native *Tk*-MGMT. GdnHCl unfolded *Tk*-MGMT completely and reversibly. The far-UV CD spectra of AdaC in these solvents are similar to those of *Tk*-MGMT except for TFE. In the presence of TFE, *Tk*-MGMT unfolded into an α-helical conformation, while AdaC aggregated.

Alcohols can stabilize a non-native (typically α-helical) protein conformation (*6*). Theoretical data using a lattice model imply a mechanism for the stabilization of the α-helical conformation, showing that the effect of alcohol is mainly to weaken non-local hydrophobic interactions and, conversely, to enhance the local α-helical interactions (*16*). In particular, TFE is a stronger denaturant of

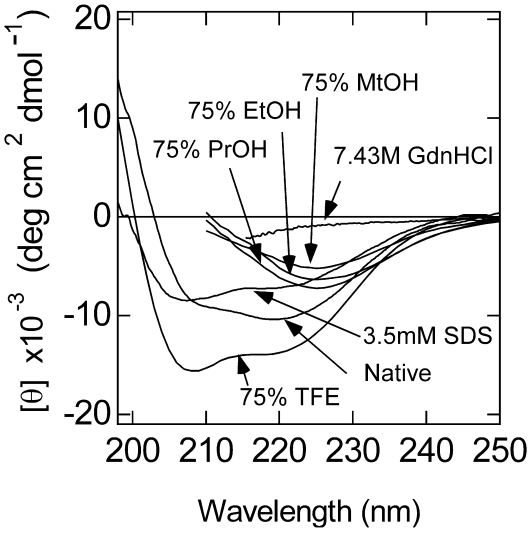

Fig. 3. **Far-UV CD spectra of native and unfolded *Tk*-MGMT in various chemical solutions.**

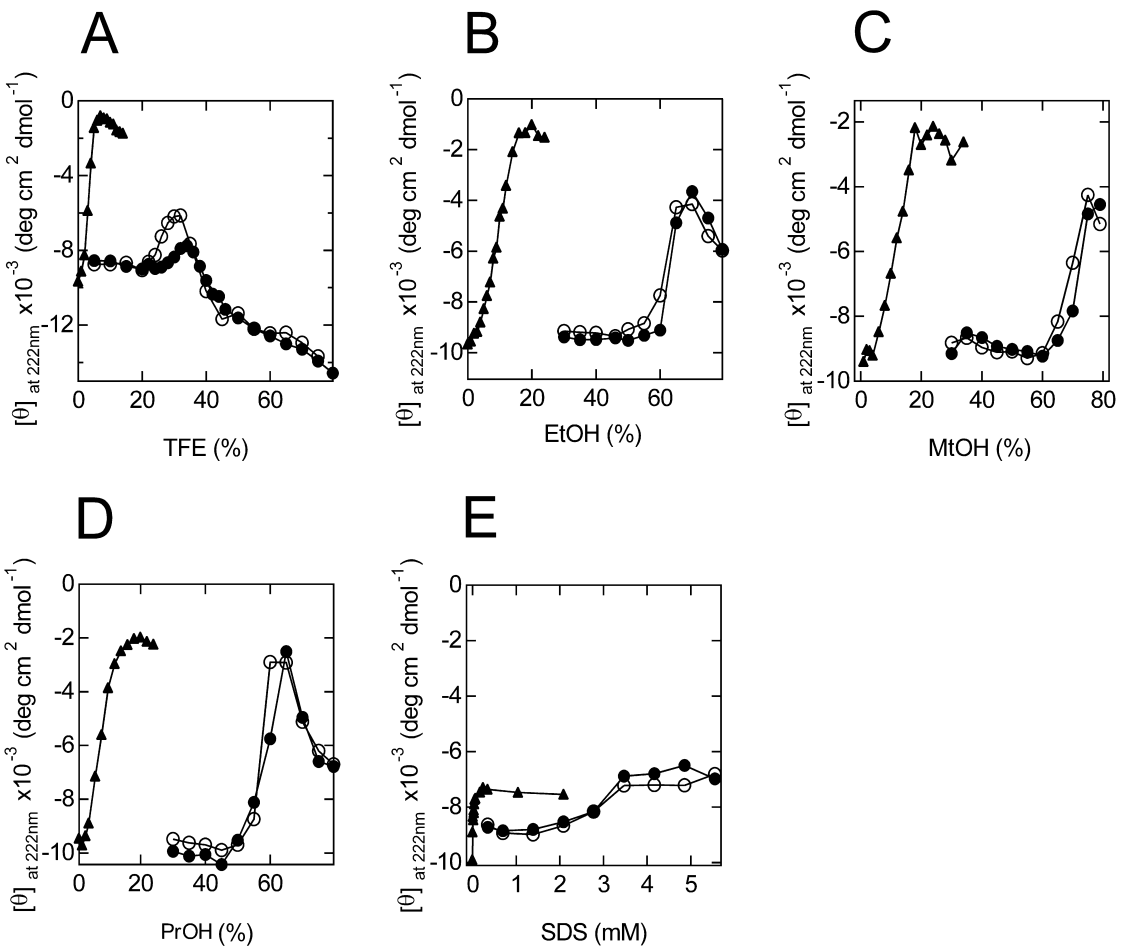

Fig. 4. **Conformational transitions of wild-type *Tk*-MGMT, E93A, and AdaC in various chemical solutions as measured by CD at 222 nm.** (A), TFE; (B), EtOH; (C), MtOH; (D), ProOH; (E), SDS. Closed circles, wild-type *Tk*-MGMT; open circles, E93A; closed triangles, AdaC.

proteins than non-halogen alcohols (*17*). The CD spectra of the TFE-induced unfolded states of *Tk*-MGMT showed typical α-helical structures, while other non-halogen alcohols caused to the formation of aggregates (Fig. 3). SDS-induced denaturation involves other mechanisms. Chemical denaturants such as urea and GdnHCl or organic solvents unfold proteins at molar concentrations, but SDS inactivates proteins at much lower concentrations, as low as 10 mM. Several models have been proposed for SDS binding to the denatured proteins, such as the rod-like particle and the α-helix and coil models (*18*). SDS interacts with proteins via interactions between the sulfate group of SDS and positively-charged amino acid side chains or bulky hydrophobic side chains (*19*). The far-UV CD spectrum of *Tk*-MGMT in SDS seems similar to that of the native state. However, no signal was observed in the near-UV CD region (data not shown), indicating that the SDS-induced unfolded state of *Tk*-MGMT contains several secondary structures, but is fully unfolded in terms of tertiary structure.

Stability to Various Chemical Solutions—We investigated the resistance of *Tk*-MGMT and AdaC to organic solvents and detergents. Furthermore, we investigated the resistance of E93A, which is destabilized by 4.2 kJ mol^{-1} and unfolds one order of magnitude faster than wild-type *Tk*-MGMT (*11*). To understand the reason for the stability in the presence of these solvents, we discriminated between the kinetic and equilibrium properties of the stability in the presence of these solvents.

Figure 4 shows the conformational transitions in various chemical solutions. The native CD spectrum of *Tk*-MGMT was retained after incubation of the protein for 30 min at 30°C in 20 mM TrisHCl (pH 8.0) containing concentrations of *ca.* 60% MtOH, 60% EtOH, 40% PrOH, 25% TFE and 1.5 mM SDS. The native spectrum of AdaC was retained at *ca.* 5% MtOH, 5% EtOH, 3% PrOH, 2% TFE, and 0.01 mM SDS. These results indicate that *Tk*-MGMT is considerably more stable in various unfolding solutions than AdaC, although these chemical conditions affect protein conformations in different ways. The fact that *Tk*-MGMT is tolerant to irreversibly-denaturing solutions such as EtOH, PrOH, MtOH, and SDS implies that *Tk*-MGMT is stabilized by kinetics rather than by equilibrium in these solutions. A comparison of stability between wild-type *Tk*-MGMT and E93A indicates that the stability of E93A does not differ from that of wild-type *Tk*-MGMT.

The kinetics of unfolding was investigated precisely using TFE and SDS. Figure 5 shows $t_{1/2}^{unf}$ of wild-type *Tk*-MGMT and E93A as a function of TFE and SDS

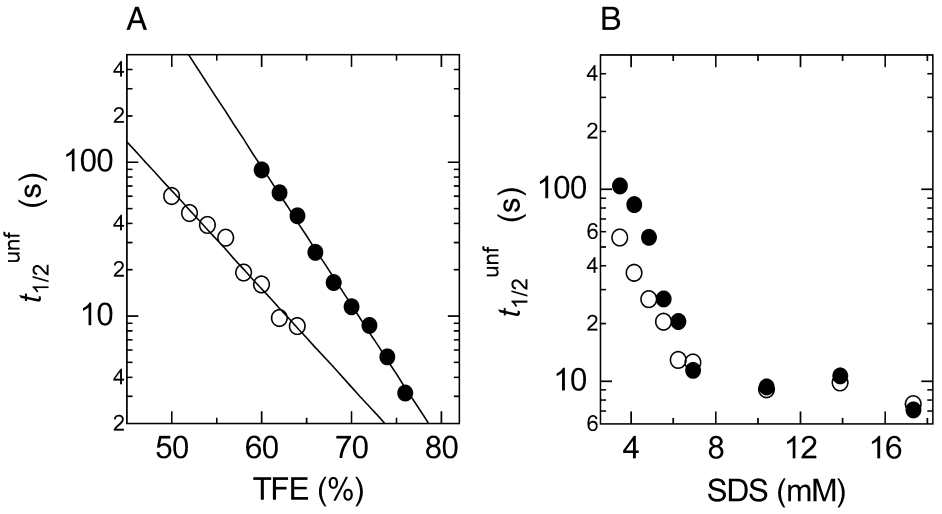

Fig. 5. **The logarithm of $t_{1/2}^{unf}$ as a function of the concentrations of TFE (A) and SDS (B).** The $t_{1/2}^{unf,H_2O}$ values were calculated according to Eq. 3. Solid lines represent the results of nonlinear regression analysis according to Eq. 3. Closed circles, wild-type *Tk*-MGMT; open circles, E93A.

concentration. The $t_{1/2}^{unf}$ values were found to be linearly dependent on the concentration of TFE, but not SDS (Fig. 4). Using the data for TFE, the $t_{1/2}^{unf,H_2O}$ values for wild-type *Tk*-MGMT were calculated by extrapolating $t_{1/2}^{unf}$ back to a zero-concentration solution. As expected, the $t_{1/2}^{unf,H_2O}$ values using TFE was very large ($2.3 \pm 0.7 \times 10^7$ s), as was found using GdnHCl ($4.5 \pm 2.4 \times 10^6$ s). On the other hand, $t_{1/2}^{unf}$ for E93A was smaller than that of wild-type *Tk*-MGMT. The $t_{1/2}^{unf,H_2O}$ values for E93A was calculated to be $1.0 \pm 0.6 \times 10^5$ s, which is one order of magnitude smaller than that of wild-type *Tk*-MGMT. Although the unfolding rate of AdaC is too fast to be determined using our system (<1 s), we were able to show that the unfolding of wild-type *Tk*-MGMT and E93A is much slower than that of AdaC.

The $t_{1/2}^{unf}$ values reached a plateau above 7 mM SDS. This is because the denaturing effect of SDS increases dramatically above the critical micelle concentration, which is around 7 mM in water (*20*). As $t_{1/2}^{unf}$ does not depend on linearly depend on the SDS concentration, $t_{1/2}^{unf,H_2O}$ values could not be calculated. Although we did not determine the $t_{1/2}^{unf}$ values of AdaC, we found that the $t_{1/2}^{unf}$ values of *Tk*-MGMT are much larger than that of AdaC. Previously, there has been only one report studying the unfolding kinetics using the S6 protein (*21*). The $t_{1/2}^{unf}$ of S6 exhibited a plateau above 2 mM SDS, where $t_{1/2}^{unf}$ was 0.23–0.35 s. The $t_{1/2}^{unf}$ of S6 is much smaller than that of *Tk*-MGMT. These results suggest that *Tk*-MGMT exhibits slow unfolding kinetics in SDS, which denatures proteins irreversibly. The $t_{1/2}^{unf,H_2O}$ of E93A in the plateau region is no different from that of wild-type *Tk*-MGMT, but $t_{1/2}^{unf}$ is smaller than wild-type *Tk*-MGMT below 7 mM (Fig. 5B).

Structural Features of Slow-Unfolding Kinetics—Tk-MGMT has a unique ion-pair network in the protein interior that is spread among three helices and between the two major domains (Arg 50–Glu 93–Arg 139) (*11*). Mutational studies of *Tk*-MGMT indicate that this ion-pair network contributes to the equilibrium and kinetic stability of *Tk*-MGMT when using GdnHCl (*11*). We investigated the equilibrium and kinetic stability of mutant E93A to understand whether this network affecting the equilibrium and the kinetic stabilities with respect to GdnHCl also contributes to stability against organic solvents and SDS. There was a significant difference in the kinetics between wild-type *Tk*-MGMT and E93A. These results suggest that the ion-pair network that affects stability against GdnHCl also contributes to stability against organic solvents and SDS. It has been suggested that electrostatic interactions contribute to the slow unfolding kinetics of hyperthermophilic proteins (*22–24*). Therefore, the slow unfolding kinetics of *Tk*-MGMT in these chemical solutions may be due to electrostatic features.

CONCLUSION

This report shows that a hyperthermophilic protein, *Tk*-MGMT, possesses higher kinetic and equilibrium stability than mesophilic proteins. The half time for unfolding of *Tk*-MGMT calculated using GdnHCl was $4.5 \pm 2.4 \times 10^6$ s, which is two orders of magnitude larger than the values for previously reported mesophilic proteins. Moreover, *Tk*-MGMT was also stable in various chemical solutions including EtOH, MtOH, PrOH, TFE, GdnHCl, and SDS. If this is a property common to hyperthermophilic proteins, a number of applications in various industries appear possible. There has recently been increasing interest in the enzymatic biotransformation in organic solvents, such as for lipase and peptidase (*25*, *26*). However, the main disadvantage of using enzymes in organic solvents is that many enzymes are rapidly denatured by these solvents. The kinetic stability of hyperthermophilic proteins under extreme conditions should aid in understanding the principles of protein folding as well as in the application of proteins in industry.

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