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SHORT COMMUNICATION

Extreme Free Energy of Stabilization of Taq DNA Polymerase

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ABSTRACT We have examined the chemical denaturations of the Klenoq and Klenow large-fragment domains of the Type 1 DNA polymerases from *Thermus aquaticus* (Klenoq) and *Escherichia coli* (Klenow) under identical solution conditions in order to directly compare the stabilization energetics of the two proteins. The high temperature stability of Taq DNA polymerase is common knowledge, and is the basis of its use in the polymerase chain reaction. This study, however, is aimed at understanding the thermodynamic basis for this high-temperature stability. Chemical denaturations with guanidine hydrochloride report a folding free energy (ΔG) for Klenoq that is over 20 kcal/mol more favorable than that for Klenow under the conditions examined. This difference between the stabilization free energies of a homologous mesophilic–thermophilic protein pair is significantly larger than generally observed. This is due in part to the fact that the stabilization free energy for Klenoq polymerase, at 27.5 kcal/mol, is one of the largest ever determined for a monomeric protein. Large differences in the chemical midpoints of the unfolding (C_m) and the dependences of the unfolding free energy on denaturant concentration in the transition region (m -value) between the two proteins are also observed. Measurements of the sedimentation coefficients of the two proteins in the native and denatured states report that both proteins approximately double in hydrodynamic size upon denaturation, but that Klenoq expands somewhat more than Klenow. *Proteins* 2004;54:616–621. © 2004 Wiley-Liss, Inc.

Key words: chemical denaturation; thermophilic; Taq polymerase; protein unfolding; Klenow polymerase; sedimentation coefficient

INTRODUCTION

Taq DNA polymerase is both structurally and functionally homologous to *Escherichia coli* Pol 1 DNA polymerase,^{1–5} yet Taq is known to retain at least partial activity at temperatures up to 97.5°C.^{1,2} Due to its uses in the polymerase chain reaction and DNA sequencing, Taq polymerase is one of the most widely used biotechnological

reagents in the world. However, the molecular basis for Taq's thermal stability is only partially understood,^{3–5} and its free energy of stabilization has never been previously examined. Elucidations of both the thermodynamic and molecular origins for the stability of thermophilic proteins continue to be separate but overlapping areas of active research and debate.^{6–9} Taq and *E. coli* DNA polymerases are both single polypeptide chains comprised of three structure/function domains: a polymerization domain, a proofreading domain (inactive in Taq), and a 5' nuclease domain. Removal of the 5' nuclease domain yields the Klenoq and Klenow "large fragments" of each polymerase, both of which are active polymerases in their own right. Klenoq and Klenow polymerases have highly homologous tertiary structures (see Fig. 1). The thermal stabilities of Taq and other thermophilic polymerases are generally measured by monitoring the loss of enzymatic activity as a function of incubation time at an elevated temperature.^{1,2} Although such measurements provide a useful and reproducible way of comparing relative stabilities, there is no way to connect them to actual thermodynamic properties. In this study, we have directly compared the chemical denaturations of Klenoq and Klenow polymerases under identical solution conditions. The results indicate that Klenoq has one of the highest free energies of unfolding yet determined for any monomeric protein, and that the stabilization free-energy difference between Klenoq and Klenow is one of the largest yet characterized for a homologous mesophilic–thermophilic protein pair.

Abbreviations: CD, circular dichroism; GdnHCl, guanidine hydrochloride; Klenow, the "large fragment" domain of *Escherichia coli* Pol 1 DNA polymerase; Klenoq, the "large fragment" domain of *Thermus aquaticus* Type 1 DNA polymerase (Taq polymerase).

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Fig. 1. Structures of Klenow (1KFD) and Klenotaq (1KTQ) polymerases displayed as ribbon diagrams.^{4,29}

MATERIALS AND METHODS

Proteins were purified as described previously¹⁰ and were used within 2–4 weeks of purification. For chemical denaturations, proteins were first dialyzed into 10 mM Tris, 125 mM KCl, and 5 mM MgCl₂ at pH 7.5. Protein samples (concentration 0.1 mg/mL) were incubated with different concentrations of GdnHCl (or urea) for 1 h, then transferred to a 2 mm pathlength quartz cuvette. Equilibrium unfolding was complete within 5 min for both proteins at room temperature (data not show). CD spectra from 215 to 225 nm were recorded using an AVIV Model 202 CD spectrophotometer. Protein concentrations were determined using the Bradford assay.¹¹ GdnHCl stock concentrations were determined by refractive index as described by Nozaki.¹² Urea concentrations were determined as described by Pace.¹³ For each titration, four independent curves consisting of CD signals at 218, 219, 220, and 221 nm were plotted versus denaturant concentration for individual analysis.

The raw CD signal (in millidegrees) of each sample at 218, 219, 220, and 221 nm was transformed into molar ellipticity ($\Delta\epsilon$),¹⁴ and denaturation curves at each wavelength were analyzed using the nonlinear form of the linear extrapolation method¹⁵:

$\Delta\epsilon$

$$= \frac{(\Delta\epsilon_N + m_N[D]) + (\Delta\epsilon_U + m_U[D])e^{-(\Delta G^\circ_{\text{unfolding}}/RT + m_G[D]/RT)}}{1 + e^{-(\Delta G^\circ_{\text{unfolding}}/RT + m_G[D]/RT)}}. \quad (1)$$

Here, $\Delta\epsilon$ is the molar ellipticity at a given wavelength (the dependent variable), $[D]$ is the molar denaturant concentration (the independent variable), $\Delta\epsilon_N$ is the y-intercept of the native state baseline, m_N is the slope of the native state baseline, $\Delta\epsilon_U$ is the y-intercept of the unfolded state baseline, m_U is the slope of the unfolded state baseline, $\Delta G^\circ_{\text{unfolding}}$ is the extrapolated free energy of unfolding in the absence of denaturant, R is the gas constant, T is the temperature in Kelvin, and m_G (the “ m -value”) is the slope

of the calculated dependence of ΔG on $[D]$. Data were fit using the program KaleidaGraph (Synergy Software, Inc).

Fluorescence titrations for Klenotaq were performed as described for CD titrations, except that denaturation was monitored by following the change in the intrinsic fluorescence of the protein as a function of added GdnHCl. Excitation was at 282 nm. The emission wavelength shifts as the protein denatures, so denaturations were followed by monitoring the emission at 335 and by monitoring the wavelength shift. Klenotaq fluorescence at 335 nm decreases with added GdnHCl and $\Delta G_{\text{unfolding}}$ was determined using the decreasing equivalent of Eq. (1).

Reversibility of denaturation was checked after removal of GdnHCl from the denatured polymerases by stepwise dialysis (in 1 *M* steps) back to 0 *M* GdnHCl, followed by retitration of the proteins with GdnHCl. Klenotaq denaturation was fully reversible. Klenow denaturation was only partially reversible. A variety of different renaturation procedures were examined for Klenow, including rapid dialysis (nonstepwise), removal of GdnHCl by Sephadex chromatography, removal of GdnHCl by centrifuged desalting columns, and incubation of “renatured” Klenow for up to 2 weeks in 0 *M* GdnHCl. All of these procedures yielded the same results. It should be noted that precipitation and loss of protein occurs in solutions of both polymerases anytime they are dialyzed, including during removal of GdnHCl. Precipitated protein was always removed by centrifugation.

Sedimentation coefficients were measured in a Beckman Optima XL-A analytical ultracentrifuge. Polymerases were dialyzed against a buffer nearly identical to that used in the denaturation titrations (10 mM Tris, 125 mM KCl, 5 mM MgCl₂, pH 7.9), and experiments were conducted in the absence and presence of GdnHCl. Protein concentrations were determined using the Bradford method.¹¹ To measure the sedimentation coefficients of the denatured polymerases, the proteins were incubated with 5.2 *M* buffered GdnHCl for 1 h. The sample and reference sectors of Epon charcoal-filled double-sector cells were loaded, respectively, with 400 μ L of 5 mM protein and 425 μ L of buffer with matching GdnHCl concentration. All velocity runs were conducted at 20°C in an An-60 Ti 4-cell rotor at 38,000 rpm. In the absence of GdnHCl, the velocity experiments were run for 3.5 h, and at 5.2 *M* GdnHCl, they were run for 16 h. The absorbance was monitored at the λ_{max} for each protein solution, which varied between 275 and 282 nm. Twenty absorbance scans with a 0.004-cm step size were recorded at 10-min intervals in the absence of GdnHCl and at 40-min intervals for the 5.2 *M* GdnHCl experiments. Data were analyzed using the modified Fujita–MacCosham model for a single species in the program Svedberg.^{16,17} Solution density measurements were performed at 20°C using an Anton-Paar DMA 58 digital densitometer. The partial specific volumes of Klenotaq and Klenow were measured previously.¹⁸ The measured densities and partial specific volumes, along with the relative solution viscosities, were used to correct the sedimentation coefficients to $s_{20,w}$ values. Viscosity measurements were conducted at 20°C using a calibrated

Fig. 2. Guanidine hydrochloride denaturations of Klenow (squares, leftmost set of curves) and Klentaq (circles, rightmost set of curves) polymerases at 25°C under identical buffer conditions in 10 mM Tris, 125 mM KCl, and 5 mM MgCl₂ at pH 7.5. Individual data points and best fits to the linear extrapolation model¹⁵ are shown for the CD signals at 218, 219, 220, and 221 nm for each polymerase.

Cannon-Manning semimicro kinematic viscometer. Effective Stokes radii (R_s) for the polymerases were calculated from the measured $s_{20,w}$ values using the equation $R_s = MW(1 - \bar{v}\rho)/6N\pi\eta s_{20,w}$, where MW is the molecular weight, $\bar{v}$ is the partial specific volume, ρ is the solvent density, N is Avogadro's number, and η is the solvent viscosity.¹⁹

RESULTS AND DISCUSSION

Unfolding Free Energy of Klentaq Polymerase

Figure 2 shows the GdnHCl denaturations of Klentaq and Klenow polymerases monitored by CD spectrophotometry. The titration curves were analyzed using the linear extrapolation method,¹⁵ and the determined unfolding free energies ($\Delta G_{\text{unfolding}}$) and associated values are reported in Table I. The $\Delta G_{\text{unfolding}}$ for Klentaq is one of the highest ever reported for a monomeric protein. Search of an online thermodynamic protein stability database reveals that only about 10 of 1278 published studies report proteins with higher unfolding free energies, and almost all of these are proteins with multiple subunits.²⁰ The free energy of subunit assembly in multimeric proteins is often quite large and can dramatically increase the overall $\Delta G_{\text{unfolding}}$ for multimeric proteins.

The chemical denaturation of Klentaq is reversible, and the protein denatures in a single transition that is fit well with a two-state denaturation model. Monitoring the GdnHCl denaturation by intrinsic fluorescence yields the same $\Delta G_{\text{unfolding}}$ as monitoring by CD (Fig. 3, Table I). The native-state baseline for the denaturation (between 0 and ~3.8 M GdnHCl) is not perfectly linear and seems to drift downward prior to the unfolding transition. This may be indicative of a more complex unfolding process involving more than two states. This deviation, however, is too subtle to be effectively fit to more complex unfolding models, so only two-state fitting results are reported. Also, cutting the unfolding data at almost any position along this baseline does not alter the fitted unfolding free energy

outside of error. The strongest argument for a two-state unfolding process for Klentaq is the agreement between the unfolding free energies determined fluorometrically and using CD. It is certainly possible, however, that the native-state structure "drifts," or that a slightly altered structural state becomes populated at low GdnHCl concentrations prior to the unfolding of the protein.

Very few thermophilic proteins exhibit reversible denaturation.^{9,21} While the GdnHCl denaturation of Klentaq is reversible, its thermal denaturation is not.²² Also, interestingly, Klentaq is not denatured by urea (data not shown). Specific resistance to urea denaturation has also been observed for a thermophilic cellulase from *Thermomonospora fusca*,²³ where GdnHCl denatured the protein "normally," whereas urea required >24 days of incubation to denature the protein. Klentaq has begun to degrade on its own during normal storage at 4°C after this amount of time, so similar long-duration urea denaturations were not attempted. To assay whether the more electrostatic character of GdnHCl could be responsible for the difference between the two denaturants, Klentaq was titrated with urea in the presence of 2 M KCl, but even at high salt, urea had no detectable effect on the CD spectrum of Klentaq (data not shown). Urea will, however, denature Klentaq at highly elevated temperatures (>75°C; data not shown).

The large $\Delta G_{\text{unfolding}}$ of Klentaq is an interesting finding; however, it may be a fortuitous result of the fact that the stability of monomeric proteins as large as Klentaq (62.4 kDa) are relatively rarely characterized thermodynamically due to the known problems with reversibility and multidomain unfolding generally encountered with larger proteins.^{24,25} Examination of energetic trends in a large collection of protein stability studies has suggested that while there are numerous exceptions, larger proteins will generally tend to have larger values of $\Delta G_{\text{unfolding}}$.⁷ Mesophilic–thermophilic protein pairs are generally exceptions to this correlation, since they are usually similarly sized proteins that often have different values of $\Delta G_{\text{unfolding}}$, as discussed further below. Klenow, for example, at 68 kDa, is slightly larger than Klentaq, yet is far less thermodynamically stable, as is also discussed below.

Difference in Unfolding Free Energies Between Klentaq and Klenow

The $\Delta G_{\text{unfolding}}$ values determined from chemical denaturations show that the free energy of stability of Klentaq is approximately 6 times higher than that of Klenow (Table I). Thermophilic proteins do not always have higher stabilization free energies ($\Delta G_{\text{unfolding}}$ values) than their homologous mesophilic counterparts. Other thermodynamic explanations can account for an increase in T_m , but recent surveys of the growing pertinent database show that the "higher T_m = higher $\Delta G_{\text{unfolding}}$ " correlation is true in the majority of cases.^{7,9,21,23} A recent survey and analysis by Nussinov and associates of published studies of mesophilic and thermophilic homologous protein pairs found a maximal $\Delta\Delta G_{\text{unfolding}}$ of 10 kcal/mol (corresponding to a mesophilic–thermophilic pair of archaeal his-

TABLE I. Fitted Parameter Values for the Denaturations of Klentaq and Klenow Polymerases

Protein	$\Delta G_{\text{unfolding}}$ (kcal/mol ⁻¹)	C _m (molar) denaturation midpoint	<i>m</i> -value (kcal/mol ⁻¹ M ⁻¹)
Klentaq ^a	27.5 ± 3.5	4.3 ± 0.1	6.4 ± 0.9
Klentaq (reversibility) ^b	27.1 ± 1.0	4.2 ± 0.2	6.5 ± 0.4
Klentaq (fluorescence) ^c	25.1 ± 9.8	4.3 ± 0.2	5.7 ± 0.7
Klenow ^d	4.3 ± 0.9	1.5 ± 0.1	3.1 ± 0.6
Klenow (urea) ^b	5.0 ± 0.3	3.6 ± 0.1	1.5 ± 0.1
Klenow (reversibility) ^b	1.7 ± 0.2	2.0 ± 0.1	1.2 ± 0.1

^aValues are means of 20 curves.

^bValues are means of 8 curves.

^cValues are means of 2 curves.

^dValues are means of 24 curves.

Fig. 3. Guanidine hydrochloride denaturation of Klentaq polymerase monitored by fluorescence. Shown is the shift in the wavelength of the emission maximum for the intrinsic protein fluorescence as the protein denatures. Excitation is at 282 nm. The line shows the best fit to a corresponding form of the linear extrapolation model.¹⁵

tones), and a mean $\Delta\Delta G_{\text{unfolding}}$ of less than half that.²¹ The $\Delta\Delta G_{\text{unfolding}}$ of 23 kcal/mol between Klentaq and Klenow is significantly higher than this, making the Klentaq–Klenow protein pair one of the most extreme examples of the “higher T_m = higher $\Delta G_{\text{unfolding}}$ ” model for explaining temperature stabilization.

Current surveys of mostly mesophilic proteins,⁷ and of mesophilic–thermophilic protein pairs,²¹ also indicate that there is a general correlation between increased T_m for any protein and increased $\Delta G_{\text{unfolding}}$, although there are clear and notable exceptions. The $\Delta\Delta T_m$ between Klentaq and Klenow is ~60°C.²² The largest $\Delta\Delta T_m$ in the survey by Nussinov and associates is 40°C and corresponds to the same pair of archaeal histones.²¹ The data herein show that Klentaq and Klenow follow this general correlation (high $\Delta\Delta T_m \approx$ high $\Delta\Delta G_{\text{unfolding}}$), but that they are quantitatively at the extreme edge of mesophilic–thermophilic protein pairs thus far characterized.

The determined $\Delta\Delta G_{\text{unfolding}}$ between Klentaq and Klenow of course depends on the separate determinations of the $\Delta G_{\text{unfolding}}$ for each protein individually. Although GdnHCl and urea unfolding of Klenow yield the same

TABLE II. Hydrodynamic Sizes of Native and Denatured States of Klentaq and Klenow Polymerases

Protein (GdnHCl)	$s_{20,w}$ ($\times 10^{-13}$ s)	R_s (Å)
Klentaq (0 M GdnHCl)	4.08 ± 0.03 ^a	35.3 ^a
Klentaq (5.2 M GdnHCl)	1.98 ± 0.01	72.6
Klenow (0 M GdnHCl)	4.59 ± 0.06 ^a	35.3 ^a
Klenow (5.2 M GdnHCl)	2.46 ± 0.01	65.8

^aNative state values are from Joubert et al.¹⁸

$\Delta G_{\text{unfolding}}$, neither denaturation is fully reversible. While removal of GdnHCl recovered 89% of the original CD signal for Klenow, redensaturation yielded only ~40% of the original unfolding free energy. The $\Delta G_{\text{unfolding}}$ for Klenow in Table I should therefore be treated with caution and regarded as an apparent or approximate measure of the stability of this polymerase. This situation, where the thermophilic protein denatures reversibly while the mesophilic protein does not, is the exact opposite of expectation.

Size Changes Upon Denaturation

The expansion in size upon denaturation of Klentaq and Klenow was characterized using velocity sedimentation. Values of $s_{20,w}$ for Klentaq and Klenow in their native (0 M GdnHCl) and denatured (5.2 M GdnHCl) states are reported in Table II, along with conversion of $s_{20,w}$ values into the effective Stokes radii for equivalent spheres. The $\Delta s_{20,w}$ between the native and denatured states is 2.1 for both polymerases (Table II). The calculation of the effective Stokes radii includes contributions from the differing molecular weights of the two polymerases (Klentaq is 62,400, Klenow is 68,100) and indicates that both polymerases approximately double in hydrodynamic size, with Klentaq expanding about 20% more than Klenow.

The data in Figure 2 and Table I, besides presenting the unusually large difference in the stabilization free energies of Klentaq and Klenow polymerases, also show that the denaturation midpoint (C_m) is much higher for Klentaq, and that the denaturation m -value for Klentaq is approximately double that for Klenow. The m -value is the dependence of the unfolding free energy on denaturant concentration in the transition region of the denaturation curve (the function of the slope of the transition). The

m -values for many proteins have been shown to correlate with the amount of protein surface area exposed upon unfolding.^{26,27} Since the folded structures of KlenTaq and Klenow are nearly identical (see Fig. 1), the doubling of the m -value suggested that the denatured state of KlenTaq may be significantly more expanded than that of Klenow. The hydrodynamic data reported in Table II indicate that KlenTaq does indeed expand more during denaturation than Klenow, but it does not expand by twice as much as Klenow. Results in other systems have also revealed exceptions to the strict quantitative interpretation of the relative m -value as being solely a reflection of the relative size of the denatured ensemble.²⁸ It is also notable that Klenow and KlenTaq are both considerably larger than any of the proteins used to demonstrate this general correlation,^{26,27} and that the m -values for Klenow and KlenTaq polymerases are both considerably lower than would be expected from an extrapolation of that database to proteins the size of KlenTaq and Klenow.²⁷ While in some systems the predominant underlying molecular determinant of the m -value may be the change in surface area upon denaturation, there seem to be significant additional, and as yet unidentified, contributions to this experimental parameter in other systems. It is also certainly possible that despite the apparent two-state nature of the denaturation of KlenTaq polymerase, intermediate states are populated during its chemical unfolding. If such were the case, both the determined m -values and $\Delta G_{\text{unfolding}}$ values would be underestimates of the true values, which would make KlenTaq an even more thermodynamically extreme system.

CONCLUSIONS

The search for the thermodynamic origins of enhanced thermal stability is paralleled by studies seeking molecular- and atomic-level explanations for this behavior.^{6,8,9} The current consensus on the molecular origins of hyperthermal stability is that there is no consensus: that each protein is a unique case, and while increased ionic interactions are the noncovalent feature most prevalently correlated with thermophily, any particular thermophilic protein may use a combination of multiple molecular strategies.^{6,8,9} The structural comparisons between Taq/KlenTaq and Klenow from two different laboratories focus on different aspects of the two structures. Steitz and associates indicate that the structures reveal four additional internal hydrogen bonds in Taq relative to Klenow, and an exchange of two ionic interactions for two hydrophobic interactions in the polymerase domain.³ Waksman and associates report a decrease in the number of unfavorable electrostatic interactions in KlenTaq, a "global reorganization of the charge distribution" leading to a greater net electrostatic stabilization, and a slight increase (1.0–1.6%) in the fraction of buried hydrophobic surface area in KlenTaq relative to Klenow.⁴ These structural rearrangements, or some subset or global consequence of them, presumably wholly or partially accounts for the sextupling of the free energy of stabilization of KlenTaq relative to Klenow. The data of this preliminary study provide ener-

getic reference states for further studies of the thermodynamic and molecular origins of the enhanced thermal stability of Taq polymerase. Further studies aimed at obtaining full Gibbs–Helmholtz stability curves for both polymerases, and mutagenic analysis of the molecular origins of the stability differences between the polymerases, are in progress in our laboratory.

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