Comparative thermal denaturation of Thermus aquaticus and Escherichia coli type 1 DNA polymerases

Irene Karantzeni
*Louisiana State University*

Carmen Ruiz
*Louisiana State University*

Chin Chi Liu
*Louisiana State University*

Vince J. Licata
*Louisiana State University*

Follow this and additional works at: [https://digitalcommons.lsu.edu/biosci_pubs](https://digitalcommons.lsu.edu/biosci_pubs)

**Recommended Citation**

This Article is brought to you for free and open access by the Department of Biological Sciences at LSU Digital Commons. It has been accepted for inclusion in Faculty Publications by an authorized administrator of LSU Digital Commons. For more information, please contact ir@lsu.edu.
Comparative thermal denaturation of *Thermus aquaticus* and *Escherichia coli* type 1 DNA polymerases

Irene KARANTZENI1, Carmen RUIZ, Chin-Chi LIU and Vince J. LICHATA2

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, U.S.A.

Thermal denaturations of the type 1 DNA polymerases from *Thermus aquaticus* (*Taq* polymerase) and *Escherichia coli* (Pol 1) have been examined using differential scanning calorimetry and CD spectroscopy. The full-length proteins are single-polypeptide chains comprising a polymerase domain, a proofreading domain (inactive in *Taq*) and a 5′ nuclease domain. Removal of the 5′ nuclease domains produces the ‘large fragment’ domains of Pol 1 and *Taq*, termed Klenow and Klentaq respectively. Although the high temperature stability of *Taq* polymerase is well known, its thermal denaturation has never been directly examined previously. Thermal denaturations of both species of polymerase are irreversible, precluding rigorous thermodynamic analysis. However, the comparative melting behaviour of the polymerases yields information regarding domain structure, domain interactions and also the similarities and differences in the stabilizing forces for the two species of polymerase. In differential scanning calorimetry, Klenow and Klentaq denature as single peaks, with a melting temperature \( T_m \) of 37 and 100 °C respectively at pH 9.5. Both full-length polymerases are found to be comprised of two thermodynamic unfolding domains with the 5′ nuclease domains of each melting separately. The 5′ nuclease domain of *Taq* denatures as a separate peak, 10 °C before the Klentaq domain. Melting of the 5′ nuclease domain of Pol 1 overlaps with the Klenow fragment. Presence of the 5′ nuclease domain stabilizes the large fragment in Pol 1, but destabilizes it in *Taq*. Both Klentaq and Klenow denaturations have a very similar dependence on pH and methanol, indicating similarities in the hydrophobic forces and protonation effects stabilizing the proteins. Melting monitored by CD yields slightly lower \( T_m \) values, but almost identical van’t Hoff enthalpy \( \Delta H \) values, consistent with two-state unfolding followed by an irreversible kinetic step. Analysis of the denaturation scan rate dependences with Arrhenius formalism estimates a kinetic barrier to irreversible denaturation for Klentaq that is significantly higher than that for Klenow.

Key words: CD, differential scanning calorimetry, protein folding, *Taq* polymerase, thermophilic.

INTRODUCTION

Full-length DNA polymerase 1 from *Thermus aquaticus* (*Taq* polymerase) is both structurally and functionally homologous with DNA polymerase 1 from *Escherichia coli* (Pol 1) [1–5], yet *Taq* is known to retain at least partial activity at temperatures up to 97.5 °C [1,2]. Owing to its uses in PCR and DNA sequencing, *Taq* polymerase is one of the most widely used biotechnological reagents in the world. However, the molecular basis for the thermal stability of *Taq* is understood only partially [3–5], and the thermodynamic basis for its stability has never been examined. Both *Taq* and *E. coli* Pol 1 full-length polymerases are single polypeptide chains comprising a polymerase domain, a 3′ exonuclease (or proofreading) domain and a 5′ nuclease domain. The proofreading domain of *Taq* is inactive [2]. Figure 1 shows the domain structure of full-length *Taq*. Removal of the 5′ nuclease domain from the full-length polymerase produces the ‘large fragment’ domains of *E. coli* Pol 1 and *Taq*, termed Klenow and Klentaq respectively [6,7]. Both Klenow and Klentaq are functional DNA polymerases on their own. Crystal structures of Klenow and Klentaq polymerases have been determined and they show a high degree of structural similarity [4,6]. Currently, there is no crystal structure for full-length *E. coli* Pol 1.

Elucidation of both the thermodynamic and structural origins of the stability of thermophilic proteins continues to be separate but overlapping areas of active research and debate [8–13]. The current consensus on the structural origins of hyperthermal stability is that there is no consensus; each protein is unique and, whereas increased ionic interactions are the non-covalent feature correlated most prevalently with thermophily, any particular thermophilic protein may use a combination of multiple molecular strategies [8,9]. As with many thermophilic proteins, the structural rearrangements in *Taq* which are believed to be associated with high temperature stability are relatively modest. The structural comparisons between *Taq*/Klentaq and Klenow from two different laboratories focus on different aspects of the two structures. Steitz and co-workers [3] indicate that the structures reveal a small number of additional internal hydrogen bonds in *Taq* relative to Klenow and also an exchange of two ionic interactions for two hydrophobic interactions in the polymerase domain. Waksman and co-workers [4] report a decrease in the number of unfavourable electrostatic interactions in the thermophilic protein, a ‘global reorganization of the charge distribution’ in the proteins leading to a higher net electrostatic stabilization in Klentaq and approx. 1–1.6% increase in the fraction of buried surface area which is hydrophobic in Klentaq relative to Klenow.

To investigate further the nature of high temperature stability of *Taq*, we directly compared the thermal melting behaviour of *Taq*/Klentaq and *E. coli* Pol 1/Klenow. The thermal stabilities of *Taq* and other thermophilic DNA polymerases have been examined previously by monitoring the loss of enzymic activity as a function of incubation time at increased temperatures. Such measurements

---

Abbreviations used: DSC, differential scanning calorimetry; DTT, dithiothreitol; Pol 1, DNA polymerase 1 from *Escherichia coli*; Klenow, the ‘large fragment’ domain of *E. coli* Pol 1; *Taq* polymerase, full-length DNA polymerase 1 from *Thermus aquaticus*; Klentaq, the ‘large fragment’ domain of *Taq* \( T_m \) melting temperature.

1 Present address: AstraZeneca R&D Boston, 35 Gatehouse Dr., Waltham, MA 02451, U.S.A.

2 To whom correspondence should be addressed (e-mail licata@lsu.edu).
provide a useful and reproducible way of comparing relative enzymic stabilities. In the present study, however, we have collected differential scanning calorimetric (DSC) denaturation data for Taq and Pol 1 DNA polymerases and their ‘large fragments’ (Klentaq and Klenow) in an attempt to understand further the basis for the enhanced temperature stability of Taq. In addition, Klenow and Klentaq have been examined as a function of pH, methanol and scan rate, and their denaturation has also been monitored by CD spectroscopy. Because both polymerases denature irreversibly, thermodynamic analysis of the data is essentially precluded. As more proteins are being examined by calorimetric and thermal denaturation methods, it is becoming clear that ‘thermal unfolding of most mesophilic and thermophilic proteins is irreversible’ [10]. Although irreversible denaturations cannot be analysed thermodynamically, it does not mean they are devoid of information. Despite their irreversibility, the results of the present study reveal information on the domain structure, domain–domain interactions, similarities in stabilizing forces and differences in the kinetic stabilization of these two species of polymerase. The results presented here are the first of their kind for either polymerase.

**EXPERIMENTAL**

Proteins were expressed and purified as described previously [14,15]. No surfactants were used during preparation, storage or calorimetry of the proteins. Calorimetric experiments were conducted within 1–2 weeks after protein purification. For each DSC experiment, proteins were dialysed against a buffer containing 10 mM K2HPO4/10 mM EDTA/2 mM dithiothreitol (DTT) at the pH values indicated for each experiment. One of the challenges in DSC is to find solution conditions under which the protein of interest denatures reproducibly without aggregating or precipitating. EDTA treatment and removal of all salt from the denaturation buffer was necessary to prevent precipitation of the polymerases after heating. EDTA treatment also eliminated irregular low-temperature heat-capacity anomalies, presumably by removing excess bivalent cations from the polymerases. Values of pH < 9.5 also caused precipitation of full-length Taq polymerase. Protein concentrations were determined using the Bradford assay [16]. Varying the protein concentrations from 0.2 to 3.0 mg/ml did not significantly alter any calorimetric value. Experiments were performed using both a MicroCal VP-DSC and a MicroCal MC-2 with a scan rate of 1 °C/min, except where indicated for the determination of the scan rate dependence of Klentaq and Klenow. Subtraction of the buffer and transition baselines yield the excess heat-capacity curves, which were normalized for the protein concentration and analysed using the MicroCal Origin DSC software, version 5.0. Reversibility was examined by rescanning both after full denaturation and after heating to the melting temperature Tm and then cooling.

Scan rate dependence was determined using the methods described by Sanchez-Ruiz et al. [17]. The scan-rate-dependent shift in Tm for denaturation was fitted to the equation:

\[
\frac{\text{scan rate}}{T_m^2} = \frac{A R}{E_a} e^{-E_a/kT_m}
\]

such that a plot of \(\ln(\text{scan rate}/T_m^2)\) against \(1/T_m\) yields a slope \(-E_a/R\), where \(E_a\) is the activation energy for denaturation, \(R\) the gas constant and \(A\) the pre-exponential factor in the Arrhenius equation.

Thermal denaturations were also performed with optical monitoring by CD on a AVIV Model 202 CD spectrometer. Spectra were recorded from 226 to 216 nm in 1 nm steps at each temperature. Protein concentration was 0.05 mg/ml in 10 mM potassium phosphate buffer (pH 9.5). For Klenow, data were collected from 5 to 71 °C in 1–3 °C intervals, with the smallest temperature steps (1 °C) in the transition region. For Klentaq, data were collected from 60 to 110 °C in 1–2 °C intervals, again with the smallest steps (1 °C) in the transition region. Protein was incubated for 5 min with stirring at each new temperature in a rectangular cuvette (1 cm path length) with a screw top seal. CD signals at 219, 220, 221 and 222 nm were used for analysis of the unfolding curves. Thermal denaturation curves were fitted to a modified form of the van’t Hoff equation, which simultaneously fits the native and denatured baselines and the transition region to obtain the Tm and \(\Delta H\) values for denaturation [18]:

\[
\Delta e = (m_n T + b_n) + (m_d T + b_d) \left( \frac{K}{1 + K} \right)
\]

where

\[
K = \exp[-\Delta H/(1 - T/T_n)/RT]
\]

Here, \(m_n\) and \(m_d\) are the slopes and intercepts of the native- and denatured-state baselines respectively and \(T\) is the temperature. Data were fitted using the program KaleidaGraph (Synergy Software, Reading, PA, U.S.A.).

**RESULTS**

We have examined the thermal denaturation of type 1 DNA polymerases from *T. aquaticus* (Taq) and *E. coli* (Pol 1). Figure 2 shows characteristic DSC scans for Taq and Pol 1 polymerases and their Klentaq and Klenow ‘large fragments’, after subtraction of the buffer baselines. The area under a DSC curve normally yields the calorimetric enthalpy of denaturation of the protein, \(\Delta H_{cal}\). The position of the peak yields the Tm for denaturation. A
wide variety of solution conditions were examined before finding identical conditions where all four proteins could be comparatively denatured directly without one or more of them precipitating (10 mM K2PO4, 10 mM EDTA and 2 mM DTT, pH 9.5). Further exploration of the denaturation of Klentaq and Klenow polymerases was also performed as a function of pH, scan rate and added methanol.

Each of the polymerases Klenow and Klentaq thermally denature as a single peak in DSC. Both the full-length polymerases, however, denature in a two-domain fashion. The excess heat-capacity curves for full-length Pol 1 and Taq can be deconvoluted into separate contributions from the 5' nuclease and large fragment domains as shown in Figure 3 and discussed further below. The fitted values for the calorimetrically determined apparent thermodynamic parameters for the denaturation of the four proteins at pH 9.5 are shown in Table 1.

The four polymerases showed very limited reversibility upon rescanning, as commonly found for proteins of this size (Taq is 94 kDa, Pol 1 103 kDa, Klentaq 62 kDa and Klenow 68 kDa). Klenow, Pol 1 and the second peak of Taq are not reversible; isolated Klentaq is partially reversible (15–30%) and the 5' nuclease peak for Taq is mostly reversible (85–100%). The problem of irreversibility in DSC is quite common [19,20], especially with proteins larger than 20 kDa and with thermophilic proteins. The total heat absorbed per mol of protein during calorimetric denaturation is a model-independent experimental value. However, because of the lack of full reversibility, the values in Table 1, with the exception of the 5' nuclease peak of Taq, must be considered as apparent thermodynamic values. Despite their limitations, the calorimetric results reveal a number of interesting features of the structural stability of the polymerases.

Klenow and Klentaq denature calorimetrically with Tm values of 37 and 100 °C respectively under identical buffer conditions at pH 9.5 and temperature scan rates of 1 °C/min. The ΔH°val for denaturation of Klentaq is approximately double that for Klenow at their respective Tm values. Since d(ΔH) = ΔCp · dT, where Cp is the heat capacity, the ΔH of any process with a positive ΔCp (such as protein unfolding) will increase with temperature. Although the results of the present study do not allow determination of ΔCp values for the polymerases, a relatively modest ΔCp value of 1.8 kcal/mol per K (1 kcal ≡ 4.184 kJ) would put the ΔH°val values for Klenow and Klentaq on the same line. Figure 2 also shows that Klentaq appears to unfold more co-operatively than Klenow (sharper peak), both as the isolated fragment and within the full-length protein.

When the full-length proteins are examined, the 5' nuclease domains of both polymerases denature at lower temperatures than their corresponding large fragments. For Taq polymerase, the 5' nuclease domain denatures as a separate peak with a Tm of approx. 89 °C. When Pol 1 is denatured, the peak for the 5' nuclease domain overlaps with the Klenow domain and the two peaks must be resolved by non-linear regression [19], as shown in Figure 3.

Denaturations of Klentaq and Klenow were further examined as a function of pH, added methanol, scan rate and by optically monitored thermal denaturation. Figure 4 shows the relationship between Tm and pH for both Klentaq and Klenow. Only a limited pH range was obtained since pH values lower than those shown for each polymerase caused precipitation of the proteins during heating. Over the ranges examined, the Tm values decreased by −3.6 °C/pH unit for Klentaq and −4.8 °C/pH unit for Klenow. Values of pH < 9.5 caused precipitation of full-length Taq during...
Denaturations of Klentaq and Klenow were also performed in the presence of methanol concentrations between 0 and 10% (0–2.5 M). Low concentrations of methanol and other organic solvents are believed to decrease the contributions of the hydrophobic effect to protein stability [21,22]. The values of $\Delta H_{\text{cal}}^\text{app}$ for each polymerase remained constant within error (less than $\pm$10%) across all methanol concentrations (results not shown). $T_m$ values for both Klentaq and Klenow decrease linearly as a function of added methanol, and are also shown in Figure 4. Klentaq is affected slightly more by the addition of methanol compared with Klenow, with values (slopes) of $-2.6$ and $-1.8 \, ^\circ\text{C/M}$ (M, molar) respectively. It should be noted that, for studies on methanol dependence, the D424A (Asp424 $\rightarrow$ Ala) mutant of Klenow was used, which is also known as KF exo$^{-}$ [23] (KF exo minus). KF exo$^{-}$ lacks the 3' exonuclease or proofreading activity, and is the predominant form of Klenow used in functional studies. Whereas both variants were not characterized under all the conditions investigated in the present study, wild-type KF and KF exo$^{-}$ were found to behave identically under those conditions where both were examined (results not shown). All the Klenow

denaturation and, hence, the pH dependence of the full-length polymerases was not characterized.

Whereas both variants were not characterized under all the conditions investigated in the present study, wild-type KF and KF exo$^{-}$ were found to behave identically under those conditions where both were examined (results not shown). All the Klenow

---

**Table 1** Fitted parameters for the thermal denaturation of DNA polymerases at pH 9.5

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H_{\text{cal}}$ (kcal/mol)$^*$</th>
<th>$\Delta H_{\text{cal}}$ (kcal/mol)$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq$^|$</td>
<td>88.9 ± 0.9</td>
<td>99.1 ± 0.1</td>
<td>147 ± 5</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>100.2 ± 0.1</td>
<td>244 ± 17</td>
</tr>
<tr>
<td>Klentaq$^|$</td>
<td>36.7 ± 0.9</td>
<td>40.4 ± 0.9</td>
<td>56 ± 9</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>36.8 ± 0.5</td>
<td>131 ± 2</td>
</tr>
</tbody>
</table>

$^*$ Only peak 1 of Taq shows reversibility after rescanning under the conditions examined. Therefore the other thermodynamic quantities in this Table should formally be considered apparent thermodynamic values.

$^\|$ For CD data, values are the means of fits to four different wavelengths as a function of temperature. As discussed in the text, $T_m$ values for CD experiments are probably lower due to an effectively significantly slower scan rate in these experiments. $T_m$ values for Klentaq and Klenow at the scan rate of 0.5 °C/min in DSC are 99.6 and 35.7 °C respectively.

$^\|$ Taq, Klentaq and Klenow excess heat capacity curves are fitted to a non-two-state model which treats each peak as a single transition but allows for asymmetry of the curve. $T_m$ and $\Delta H_{\text{cal}}$ values were determined at the scan rate of 1 °C/min and are expressed as the means ± S.D. for three experiments.

$^\|$ Pol 1 excess heat capacity curves are deconvoluted to two independent two-state transitions. Values are expressed as the means ± S.D. for three experiments.

---

---

**Figure 4** pH and methanol dependence of thermal denaturations of Klentaq and Klenow polymerases

The upper panel shows the $T_m$ versus pH dependence for Klentaq and Klenow. $T_m$ values at different concentrations of methanol are shown in the lower panel.
whereas Klentaq and Klenow were found to be scan-rate-dependent, the two polymerases. The dotted lines in the plot of Δ
\[\Delta H_{\text{app}} \text{,cal} \]
dependent changes in Tm as described in the text. The slopes of these lines provide the apparent activation energies of denaturation [17].

The top and middle panels show the Tm and Δ
\[\Delta H_{\text{app}} \text{,cal} \]
dependent changes in Tm of unfolding, ΔH
\[\Delta H_{\text{app}} \text{,cal} \] determined from the CD monitored denaturations were essentially identical with the ΔH
\[\Delta H_{\text{app}} \text{,cal} \] values for Klenow and Klentaq. This agreement suggests that the unfolding process is very nearly two-state, as agreement between calorimetric and van’t Hoff enthalpies is one of the defining characteristics of a two-state denaturation process [19,20,29]. Even though ΔH
\[\Delta H_{\text{app}} \text{,cal} \] determined from the CD monitored denaturations were essentially identical with the ΔH
\[\Delta H_{\text{app}} \text{,cal} \] values for Klenow and Klentaq. This agreement suggests that the unfolding process is very nearly two-state, as agreement between calorimetric and van’t Hoff enthalpies is one of the defining characteristics of a two-state denaturation process [19,20,29]. Even though ΔH
\[\Delta H_{\text{app}} \text{,cal} \] determined from the CD monitored denaturations were essentially identical with the ΔH
\[\Delta H_{\text{app}} \text{,cal} \] values for Klenow and Klentaq. This agreement suggests that the unfolding process is very nearly two-state, as agreement between calorimetric and van’t Hoff enthalpies is one of the defining characteristics of a two-state denaturation process [19,20,29]. Even though ΔH
\[\Delta H_{\text{app}} \text{,cal} \] determined from the CD monitored denaturations were essentially identical with the ΔH
\[\Delta H_{\text{app}} \text{,cal} \] values for Klenow and Klentaq. This agreement suggests that the unfolding process is very nearly two-state, as agreement between calorimetric and van’t Hoff enthalpies is one of the defining characteristics of a two-state denaturation process [19,20,29].

Thermal denaturations of Klenow and Klentaq were also monitored optically using CD spectrometry. The CD spectra at room temperature for both proteins reflect the high α-helical structure of the two proteins (Figure 6). As heat denatures the proteins, their secondary structure is disrupted, and the transition region of the denaturation profile can be analysed using a van’t Hoff approach (Figure 6). Simultaneous fitting of the native and denatured baselines along with fitting of the transition region to the van’t Hoff equation yields the van’t Hoff enthalpy of unfolding, ΔH
\[\Delta H_{\text{app}} \text{,cal} \] and the Tm of unfolding for the protein, as described in the Experimental section, and these values are presented in Table 1. The van’t Hoff enthalpies ΔH
\[\Delta H_{\text{app}} \text{,cal} \] determined from the CD monitored denaturations were essentially identical with the ΔH
\[\Delta H_{\text{app}} \text{,cal} \] values for Klenow and Klentaq. This agreement suggests that the unfolding process is very nearly two-state, as agreement between calorimetric and van’t Hoff enthalpies is one of the defining characteristics of a two-state denaturation process [19,20,29]. Even though ΔH
\[\Delta H_{\text{app}} \text{,cal} \] determined from the CD monitored denaturations were essentially identical with the ΔH
\[\Delta H_{\text{app}} \text{,cal} \] values for Klenow and Klentaq. This agreement suggests that the unfolding process is very nearly two-state, as agreement between calorimetric and van’t Hoff enthalpies is one of the defining characteristics of a two-state denaturation process [19,20,29].

It is noteworthy that the Δε baselines for the denatured states (upper baselines) of both proteins still exhibit negative CD intensity in the 220 nm wavelength range. Although models of random-coil conformation polypeptides are classically known to have positive CD intensity in this wavelength range, as depicted in any textbook introduction to CD, actual denatured proteins almost universally do not exhibit positive CD intensities in this region of their spectra (e.g. see [24,25]). This has often been attributed to the presence of a residual structure of the denatured state of the protein. The Δε versus temperature profiles for Klenow and Klentaq shown in Figure 6 are characteristic examples of such denaturations.

DISCUSSION

Two-domain melting of the polymerases

Although both Taq and Pol 1 polymerases contain three structural/functional domains [2,3,26], they do not denature in three peaks. In DSC, Klenow and Klentaq both denature in a single peak. Correspondence between the calorimetric and van’t Hoff ΔH values (Table 1) indicates that thermal transition is effectively two-state for both Klenow and Klentaq. The melting transition for Klentaq in DSC, however, is actually slightly more co-operative (sharper peak) than a two-state transition, possibly due to the onset of a kinetically controlled irreversible step as discussed below in the Scan rate dependence subsection. The single peak, two-state behaviour of Klenow and Klentaq indicates a tight...
I. Karantzeni and others

Figure 6 Thermal denaturations of Klenow and Klentaq monitored by CD

The top panel shows the room temperature CD spectra of the two proteins. The upper spectrum corresponds to Klenow and the lower spectrum to Klentaq. The bottom two panels show the thermal denaturations of the two proteins monitored at four different wavelengths (in both plots, data at 219, 220, 221 and 222 nm are denoted by ×, ◊, □ and ○ respectively). Lines show the fits to a modified van’t Hoff equation as described in the text.

Folding/unfolding coupling between the polymerase and the 3′ exonuclease functional domains within the large fragments. In contrast, full-length Taq polymerase denatures in two cleanly separated transitions, with the 5′ exonuclease domain denaturing approx. 10 °C before the Klentaq domain. Full-length E. coli Pol 1 denatures as a single peak, but the one that easily deconvolutes into two separate transitions. Based on the denaturation behaviour of full-length Taq, it is straightforward to assign the two melting domains in Pol 1 to the 5′ nuclease domain and the Klenow fragment.

The melting data show that the 5′ nuclease domain of Taq will in fact denature during the normal course of PCR, where the temperatures used for melting the DNA duplexes formed during each cycle are typically 94–97 °C. The 5′ nuclease domain of Taq is the only reversibly denaturing peak in DSC; thus, this domain will denature and refold during each cycle of a typical PCR run using full-length Taq. This previously unknown characteristic of the 5′ nuclease domain has been particularly fortuitous for the development of real-time PCR assays, many of which require the 5′ nuclease activity of the protein.

Inter-domain interactions

A comparison of the T_m values for the full-length proteins versus the Klentaq and Klenow fragments suggests that the unfolding domains interact in both proteins, but in opposite ways. When the 5′ nuclease domain is removed from full-length Taq, the T_m value for the Klentaq domain increases by approx. 1 °C. Thus the interaction between the Klentaq and 5′ nuclease domains in full-length Taq is slightly unfavourable for the Klentaq domain, and the Klentaq fragment is slightly more stable on its own. This observation is in agreement with an increase in the time of stability at 97.5 °C found for Klentaq versus Taq when measured using the traditional loss of activity assay [27]. Conversely, removal of the 5′ nuclease domain from Pol 1 leads to an apparent 3.6 °C T_m destabilization of the Klenow domain. Thus the presence of the 5′ nuclease domain in Pol 1 appreciably stabilizes the rest of the protein.

T_m versus growth temperature

Another interesting feature of our results is that Pol 1 and Klenow begin denaturing at or slightly above 37 °C, the optimal growth temperature of E. coli, whereas Taq/Klentaq polymerase is stable even at much higher temperatures than the 70–72 °C optimal growth range [1,28] of T. aquaticus. The melting temperatures near 37 °C for Klenow and Pol 1 indicate that additional factors such as ion binding, molecular crowding, DNA and/or nucleotide binding and possibly natural osmolytes must be acting in vivo to keep Pol 1 from denaturing at the optimal growth temperature of E. coli. Thus, unlike Taq polymerase, the intrinsic thermal stability of E. coli Pol 1 polymerase is not adequate or only marginally adequate to preserve its native state in vivo.

pH dependence

The dependence of T_m on pH is similar for both Klentaq and Klenow, both showing a modest decrease in T_m with increasing pH. The near linearity and lack of a large excursion of T_m versus pH indicate that there are no stability-linked amino acid residues with pK_v values in this range. However, deprotonation is destabilizing for both proteins. Normally, the pH-dependent shift in T_m and ΔH for a protein can be used to determine the ΔC_p, for

Figure 6 Thermal denaturations of Klenow and Klentaq monitored by CD

The top panel shows the room temperature CD spectra of the two proteins. The upper spectrum corresponds to Klenow and the lower spectrum to Klentaq. The bottom two panels show the thermal denaturations of the two proteins monitored at four different wavelengths (in both plots, data at 219, 220, 221 and 222 nm are denoted by ×, ◊, □ and ○ respectively). Lines show the fits to a modified van’t Hoff equation as described in the text.
denaturation of that protein [29]. However, the irreversible nature of these transitions (and their scan rate dependence, see below) implies that such an analysis would not be thermodynamically valid for these data, and could be misleading.

Interestingly, almost all commercially available reaction buffers used for PCR are Tris at or near pH 9 (measured at room temperature). Tris buffer has one of the largest temperature coefficients of any commonly used biological buffer, and will decrease in pH by 1–2 pH units as the temperature is increased during PCR cycling. Therefore, fortuitously, the use of Tris as the buffer of choice for PCR means that, during each high temperature portion of a normal PCR cycle, the polymerase will be additionally protected by the concomitant decrease in pH of the reaction buffer. The calorimetric experiments described in the present study were all performed in phosphate buffer, which has almost no temperature dependence. Calorimetrically, we found that, although lowering the pH from 10.5 to 8.5 increases the $T_{m}$ at pH values lower than 8.5 Klentaq precipitates on full denaturation. This raises the question of why Taq/Klentaq does not precipitate during PCR. First, full calorimetric denaturation of Taq/Klentaq involves heating the protein to 110–115 °C, temperatures far above those used in PCR. Furthermore, at lower pH values, the shift in $T_{m}$ to higher temperatures also means that the beginning of the melting transition is shifted to higher temperatures. The high temperature DNA dissociation step in PCR is generally performed at 94–97 °C. At pH 9.5, Klentaq has just started to melt by this temperature (see Figure 2), whereas at pH 8.5 its melting transition does not begin until above 98 °C.

**Methanol dependence**

The effect of low concentrations of methanol on proteins is generally attributed to the increased hydrophobicity of the solvent, which decreases the favourability of the burial of hydrophobic regions of the protein in the native state (e.g. see [21,22]). Changes in $T_{m}$ with the addition of methanol for Klentaq and Klenow (–2.6 and –1.8 °C/M respectively) are similar to the methanol dependence observed for other proteins, including lysozyme [21], lipase [30] and cytochrome c [22].

Because $d(\Delta H) = \Delta C_{p} \cdot dT$, by definition, the denaturation enthalpy will increase with temperature. Since the denaturation $T_{m}$ and $\Delta H$ of a protein can be shifted with pH [29], an effective plot of $\Delta H$ against $T_{m}$ can be constructed and extrapolated to temperatures far beyond those under which the protein is stable [22,29,31]. Convergence of extrapolated normalized denaturation enthalpies has been found to occur close to 100 °C for basically all proteins examined to date [22,29,31]. Different studies of the so-called enthalpy and entropy convergence temperatures in protein folding have led to the conclusion that, at these temperatures (which are 100 °C for $\Delta H$ and approx. 112 °C for $\Delta S$), the hydrophobic contributions to $\Delta H$ and $\Delta S$ are zero (e.g. [22,31]). Klentaq denatures exactly at the $\Delta H$ convergence temperature, whereas Klenow denatures far below it; therefore, these models might suggest that the balance of hydrophilic versus hydrophobic stabilizing forces in the two proteins might be quite different. However, methanol affects both proteins similarly and, in fact, has a larger effect on Klentaq when compared with Klenow. Thus Klentaq seems to be quite well stabilized by hydrophobic effects, perhaps more so when compared with Klenow, even at the enthalpy convergence temperature. These results might suggest that the hydrophobic contributions to $\Delta H$ are not zero for Klentaq at the enthalpy convergence temperature, or that there is a very strong favourable hydrophobic entropy effect for Klentaq at 100 °C.

**Scan rate dependence**

Thermal denaturations of both Klentaq and Klenow were found to be dependent on the rate of heating in the calorimeter. As discussed above in the Results section, the $T_{m}$ data obtained by thermal denaturation monitored by CD also indicate a scan rate effect. Scan rate dependence is generally regarded as being indicative of at least partial kinetic control of the melting process. The Lumry–Eyring reaction scheme is the most common framework used for modelling irreversible denaturation [32]. In this model, a native protein (N) undergoes two transitions: an equilibrium transition to an unfolded state (U), followed by a kinetic transition to an irreversibly denatured state (I):

$N \xrightarrow{k_{eq}} U \xrightarrow{k_{i}} I$

Support for this model for Klentaq and Klenow comes from an agreement between the calorimetric unfolding enthalpies and van’t Hoff enthalpies determined from CD denaturations, as this is the primary distinguishing characteristic of a two-state denaturation process (N $\leftrightarrow$ U) [19,20,29], whereas the irreversibility of thermal melting both in the calorimeter and the spectrophotometer and also the scan rate dependence in DSC indicate a subsequent U $\rightarrow$ I kinetic step.

Sanchez-Ruiz and co-workers [17] and other authors [35,36] have developed a number of different methods of obtaining Arrhenius plots from the scan rate dependence of irreversible denaturations. Most of these approaches begin by assuming that the rate U $\rightarrow$ I is higher than the back rate U $\rightarrow$ N and thus analyse the Arrhenius behaviour of the scan rate dependence as a single-step N $\rightarrow$ I process. Arrhenius analysis of the scan rate dependence then provides an activation energy for the full irreversible denaturation of the protein N $\rightarrow$ I. If either the N $\rightarrow$ U step or the U $\rightarrow$ I step is rate-limiting, then the obtained activation energy would be most correlated with this step, but definitive determination of the rate-limiting step is difficult. Since Sanchez-Ruiz and co-workers [17,33,34] and other authors [35,36] report more or less equivalent activation energies with each of the different Arrhenius-based analysis methods, we have demonstrated only one of these methods here (in Figure 5).

Although only a small number of proteins have been analysed by these methods, the activation energy for the irreversible denaturation of Klenow (67 kcal/mol) is similar to the majority of the proteins examined previously, whereas the $E_{a}$ for Klentaq (157 kcal/mol) is not the highest but is one of the highest $E_{a}$ values reported so far (see [36] for a summary). Within the formalism of the model, the higher activation barrier for Klentaq indicates a slower irreversible step, and implies that, in some cases, Klentaq unfolds to a large extent (i.e. an N $\leftrightarrow$ U equilibrium may be largely established) before irreversible denaturation begins [36]. Extension of similar analyses for other proteins has suggested that, in some (few) situations where the kinetic barrier to irreversible denaturation is large and its rate is low, the enthalpies derived from completely irreversible DSC curves may in fact be considered equilibrium thermodynamic parameters [36–38]. However, establishment of a widely accepted set of criteria for determining when such equivalences can be made and when they cannot is still an area of active research [38,39].

The significantly higher activation barrier for irreversible denaturation of Klentaq versus Klenow does indicate that Klentaq is significantly kinetically stabilized when compared with Klenow. In other words, temperatures at which each of them would denature (i.e. within the ranges of each of their DSC transition curves), a higher $E_{a}$ for Klentaq indicates it will take longer to irreversibly denature than Klenow. Again, since these
kinetic analyses are actually reflective of the entire process N → I, the slower process in Klenaq may be either the N → U step or the U → I step. This analysis does mean, however, that in vitro as well as in vivo Klenaq/Taq would be protected from irreversible damage at temperatures near and above its $T_m$ for longer than Klenow at temperatures near and above its $T_m$.

Concluding comments

Even though calorimetric denaturations of Taq and E. coli Pol 1 polymerases are irreversible and preclude a strict thermodynamic analysis, a parallel examination of the melting behaviours of the polymerases and their large fragments has revealed a number of similarities and differences between the two proteins. Both of the full-length polymerases melt in a two-domain fashion, with the $5'$ nucleosome domain melting separately and first. The influence of the $5'$ nuclease domain on the rest of the protein is opposite in the two proteins: it is stabilizing in E. coli Pol 1 and destabilizing in Taq. The pH and methanol dependence of the melting temperatures for Klenaq and Klenow are very similar (even though under any parallel solution condition their absolute values are separated by more than 60 °C), suggesting little or no difference in the contributions of hydrophobic forces and protonation/deprotonation effects to the stability of the two species of polymerase. Finally, Arrhenius analysis of the scan rate dependence for denaturation of Klenaq and Klenow indicates a substantially greater kinetic barrier to irreversible denaturation of Klenaq when compared with Klenow.

We thank Michael Doyle and SmithKline Beecham Pharmaceuticals for the gift of Pol 1 and Klenow expression plasmids. This work was funded by NSF grant no. 9904680 (to V. J. L.).

REFERENCES

9. Petko, G. A. (2001) Structural basis of thermostability in hyperthermophilic proteins, or “there’s more than one way to skin a cat”. Methods Enzymol. 334, 469–479