10-1-2006

Thermal stability landscape for Klenow DNA polymerase as a function of pH and salt concentration

Allison J. Richard
Louisiana State University

Chin Chi Liu
Louisiana State University

Alexandra L. Klinger
Johnson & Johnson Pharmaceutical Research & Development, Raritan

Matthew J. Todd
Johnson & Johnson Pharmaceutical Research & Development, Raritan

Tara M. Mezzasalma
Johnson & Johnson Pharmaceutical Research & Development, Raritan

See next page for additional authors

Recommended Citation

Richard, A., Liu, C., Klinger, A., Todd, M., Mezzasalma, T., & LiCata, V. (2006). Thermal stability landscape for Klenow DNA polymerase as a function of pH and salt concentration. *Biochimica et Biophysica Acta - Proteins and Proteomics, 1764* (10), 1546-1552. https://doi.org/10.1016/j.bbapap.2006.08.011

Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1764 (2006) 1546–1552
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.elsevier.com/locate/bbapap

Thermal stability landscape for Klenow DNA polymerase as a function of pH and salt concentration

Allison J. Richard [a], Chin-Chi Liu [a], Alexandra L. Klinger [b], Matthew J. Todd [b], Tara M. Mezzasalma [b], Vince J. LiCata [a,*]

[a] *Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA*
[b] *Johnson & Johnson Pharmaceutical Research and Development, L.L.C., Welsh and McKean Rds., Spring House, PA 19477, USA*

Received 21 March 2006; received in revised form 10 August 2006; accepted 14 August 2006
Available online 30 August 2006

Abstract

The thermal denaturation of Klenow DNA polymerase has been characterized over a wide variety of solution conditions to obtain a relative stability landscape for the protein. Measurements were conducted utilizing a miniaturized fluorescence assay that measures Tm based on the increase in the fluorescence of 1,8-anilinonaphthalene sulfonate (ANS) when the protein denatures. The melting temperature (Tm) for Klenow increases as the salt concentration is increased and as the pH is decreased. Klenow's Tm spans a range of over 20 °C, from 40 to 62 °C, depending upon the solution conditions. The landscape reconciles and extends previously measured Tm values for Klenow. Salt effects on the stability of Klenow show strong cation dependence overlaid onto a more typical Hofmeister anion type dependence. Cationic stabilization of proteins has been far less frequently documented than anionic stabilization. The monovalent cations tested stabilize Klenow with the following hierarchy: $NH_4^+ > Na^+ > Li^+ > K^+$. Of the divalent cations tested: Mg^{+2} and Mn^{+2} significantly stabilize the protein, while Ni^{+2} dramatically destabilizes the protein. Stability measurements performed in combined Mg^{+2} plus Na^+ salts suggest that the stabilizing effects of these monovalent and divalent cations are synergistic. The cationic stabilization of Klenow can be well explained by a model postulating dampening of repulsion within surface anionic patches on the protein.

Keywords: Pol I; Thermofluor; Denaturation; Unfolding; Cationic stabilization

1. Introduction

Solution conditions have long been known to influence protein stability. There are a small number of broad spectrum salt plus pH surveys of the thermal stability landscapes for different proteins in the scientific literature, and they demonstrate that the effects of salt and pH on protein stability are highly protein specific [1–6]. In other words, pH changes and salt additions can either increase or decrease stability, depending on the protein, and their effects are not necessarily parallel. Numerous studies of the effects of salt on protein stability exist, and for the majority of proteins examined to date, added salt stabilizes the protein [1,2,4–14]. This effect is common enough that deviations from it are notable for their rarity (e.g. [3]). Elucidation of the underlying molecular mechanisms for salt-induced stabilization of proteins, however, is still an active area of research. The responses of a specific protein to salt or pH changes are strong reflections of the nature of the protein itself, and such characterizations can reveal what types of non-covalent interactions and surface properties are linked to that protein's stability.

Klenow polymerase is the "large fragment domain" of *Escherichia coli* DNA polymerase I (Pol I), which was the first DNA polymerase discovered [15,16] and which remains the central model system for studies of DNA replication. Full length Pol I is a single polypeptide chain with three structure–function domains: a polymerization domain, a 3′ exonuclease domain, and a 5′ nuclease domain. Removal of the 5′ nuclease domain

* Corresponding author. Tel.: +1 225 578 5233; fax: +1 225 578 2597.
E-mail address: licata@lsu.edu (V.J. LiCata).

1570-9639/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2006.08.011

yields the Klenow fragment, which is a fully functional DNA polymerase [17,18]. The structure of Klenow and other DNA polymerases is often conceptually compared to a half-open right hand, with a palm, fingers, and a thumb. The palm sub-domain contains the catalytic residues [19,20].

We have previously characterized the thermal and chemical denaturation of Klenow polymerase in directly comparative studies with the thermophilic Pol I Type DNA polymerase from *Thermus aquaticus* [21,22]. Those studies revealed extremely large differences in Tm and stabilizing free energies (ΔG) between the partners in this mesophilic–thermophilic protein pair, and characterized some of the differences in the unfolding processes for the two polymerase species. In order to facilitate direct comparison, the characterizations in these previous studies were performed under identical conditions for both polymerases.

In this study we have expanded our understanding of the stability of Klenow DNA polymerase by surveying the effects of salt concentration, specific ions, and pH. Joyce and associates also previously measured Tm values for Klenow and several site directed mutants using CD spectroscopy in a solution of 20 mM potassium phosphate, 15 mM NaCl, and 15% glycerol at pH 7.0 [23]. The Tm values obtained in the studies of Joyce and associates were significantly higher (by 8–15 °C) than those obtained in our previous studies. The results of the present study clearly show that the differences between Joyce's Tm measurements and our own Tm measurements are explained by the pH-salt stability landscape.

Klenow denatures irreversibly in this assay thus precluding thermodynamic analysis of its denaturation. Most proteins denature irreversibly [24]. Despite this, characterization of the thermal and chemical denaturation of irreversibly denaturing proteins can lead to a variety of new information at the molecular level, and frequently can add to understanding the biology and physiology of the protein.

This study utilizes a miniaturized fluorescence assay that measures Tm based on an increase in the binding of 1,8-anilinonaphthalene sulfonate (ANS) to the denatured state of the protein [25–27]. We find that the Tm for Klenow in this assay ranges from 40 to 62 °C, depending on the solvent conditions, with both increases in salt and decreases in pH leading to greater stabilization. Our data also indicate that there are strong ion-specific cation effects on stability, overlaid onto a more typical Hofmeister anion type dependence. Examination of the surface electrostatic topology of Klenow has shown that the majority of the surface of the protein has a net negative charge [28]. Attenuation of surface anionic repulsion thus appears to be a reasonable explanation for the cationic stabilization of Klenow.

2. Materials and methods

2.1. Materials

Klenow DNA polymerase was overexpressed and purified according to previously described procedures [29,30]. Following purification, the protein was stored at −20 °C in 50 mM Tris, 0.5 mM DTT, and 50% glycerol, pH 7.5 until use.

2.2. ThermoFluor measurements

The ThermoFluor®[1] thermal shift assay system at Johnson & Johnson Pharmaceutical Research and Development was used to measure the thermal stability of Klenow DNA polymerase under a wide variety of buffer, pH, and salt conditions. This assay has been described in detail previously [26,27]. For reversibly unfolding proteins, this assay has been shown to be as thermodynamically reliable as conventional methods [27]. For irreversibly unfolding proteins, such as Klenow, only measured Tm values are used to deduce information about the protein. For the present study, 2 µl of each experimental buffer solution, with all components at two times the desired assay concentration, were dispensed into black 384-well polypropylene PCR microplates (Abgene), along with 2 µl of the 2× protein solution, which included the ANS fluorescent probe. Each assay well contained 0.075 mg/ml Klenow in a final volume of 4 µl. Different assay wells contained different buffers, with different salts, at different pH values, as described in the appropriate figure legends. Buffers used included 25 mM sodium acetate, MES, sodium phosphate, PIPES, HEPES, MOPS, and sodium borate (used to generate pH's from 4 to 9). Salts included 5–505 mM NaCl, 0 and 5 mM $MgCl_2$, 100 and 300 mM NH_4Cl, LiCl, and KCl, 10 and 100 mM $(NH_4)_2SO_4$, (NH_4)-PO_4, $MgSO_4$, K-PO_4, $LiSO_4$, and $NiSO_4$, and 5 mM $MnCl_2$. Each assay well also contained 0.1 mM ANS, 1 mM Tris, 2.5 mM Pipes, 0.01 mM DTT, 1% glycerol, and 0.5% DMSO. To prevent evaporation during thermal denaturation, 1 µl of silicone oil (Fluka) was dispensed over the assay solution in each well. Assay plates are then loaded onto a heating block within the ThermoFluor machine. Resident data acquisition programs are used to control the run and collection parameters. The following parameters were used for all experiments: (1) samples were heated from 25 to 85 °C, (2) samples were held for 15 s at each 1 °C increment, and imaged for 10 s during the hold time, (3) the gain was set to 2, (4) the imaging time was 10 s, (5) filtered UV light at 380–400 nm was used to excite the fluorescent probe, and (6) the fluorescence emission intensity was measured at 500 ± 25 nm using a CCD camera to simultaneously collect an intensity measurement for each of the 384 wells. For each well, the intensity was the sum of the intensity detected by all pixels within the area of a box that was 8 pixels by 8 pixels. The Tm, or midpoint of the thermal transition, can be determined either by using the maximal value of the derivative of the transition region of the melting curve, or by fitting to an extended form of the van't Hoff relationship, as described previously [27]. In the case of reversibly denaturing proteins, true thermodynamic parameters, such as the ΔH of unfolding, may be obtained from the data [27]. In irreversible systems, such as Klenow, only the Tm values are determined. Requisite controls, such as establishing that the ANS probe does not interact with the native state of the protein, have also been described previously [26,27].

2.3. CD measurements

For CD measurements at different pH values, spectra from 218 to 230 nm were recorded using an AVIV model 202 circular dichroism spectrophotometer. The protein concentration was 0.1 mg/ml, and measurements were made in 10 mM phosphate buffer with 2 mM β-mercaptoethanol. pH was adjusted by mixing monobasic, dibasic, and tribasic phosphate buffers. For CD monitored thermal denaturation in the presence and absence of $NiSO_4$, protein concentration was 0.05 mg/ml in 10 mM Tris, pH 7.9. $NiSO_4$ was 100 mM. Data were collected from 25 to 60 °C in a rectangular cuvette (1 cm path length) with a screw top seal and constant stirring. The CD signal at 222 nm was 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 for denaturation, as described previously [30].

2.4. Electrostatic potential contour

GRASP was used to calculate the electrostatic potential surface map for Klenow DNA polymerase [31]. Coordinates for Klenow (1 kfd) were obtained

[1] The ThermoFluor assay was developed by 3-Dimensional Pharmaceuticals, Inc., which has been merged into Johnson & Johnson Pharmaceutical Research and Development, L.L.C. "ThermoFluor" is a trademark registered in the United States and certain other countries.

from the Protein Data Bank. Full charges were assigned. Adjustable parameters used were default values, except that ionic strength was set at 50 mM.

3. Results

Fig. 1 shows typical denaturation curves obtained for Klenow polymerase in this study. In this figure, denaturation curves as a function of increasing NaCl concentration are shown. Data are obtained with a miniaturized fluorescence-based assay performed using the ThermoFluor® assay system at Johnson & Johnson Pharmaceutical Research and Development [26,27]. In this assay, protein unfolding is monitored by following the increase in fluorescence due to the enhanced binding of 1,8-anilinonaphthalene sulfonate (ANS) to the denatured state of the protein. The denaturation curves consist of the same three segments as more traditional denaturation curves: an initial native baseline, a transition region where the protein unfolds, and a subsequent denatured state baseline. The native and denatured state baselines for a "ThermoFluor" denaturation have more significant slopes than with many other denaturation methods due to the temperature dependence of ANS fluorescence. Controls are performed to insure that ANS does not interact with the native state of the protein (data not shown). As with any thermal denaturation curve, the midpoint of the transition region provides the Tm value. With reversibly denatured proteins, the curves can also be analyzed for denaturation enthalpy and other thermodynamic parameters [27]. Klenow polymerase does not reversibly denature, however, so our analyses here are restricted solely to the Tm. It should be noted that where it has been possible to directly compare Tm values, Thermofluor values average about 2 °C higher than CD values. This is similar to typical variations seen between CD and calorimetric Tm values.

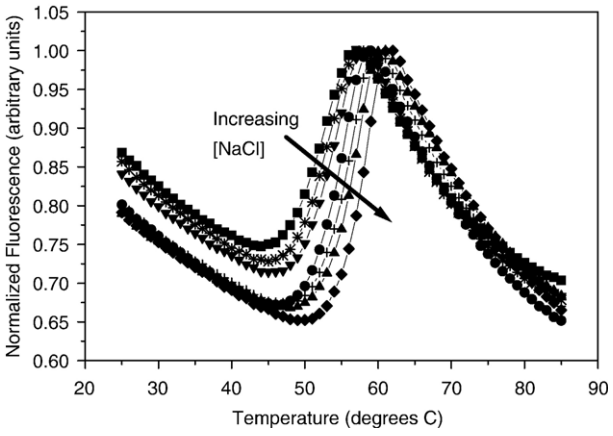

Fig. 1. Thermal melting curves for Klenow polymerase. Each individual curve, normalized for maximum fluorescence intensity, shows the thermal denaturation of Klenow at a different NaCl concentrations in 25 mM phosphate buffer, pH 7.0. The NaCl concentrations are 0 mM (squares), 50 mM (stars), 100 mM (inverted triangles), 200 mM (circles), 300 mM (crosses), 400 mM (triangles), and 500 mM (diamonds). The denaturation curves consist of the same three segments as more traditional denaturation curves: a native baseline, a transition region, and a subsequent denatured state baseline. The native and denatured state baselines for a "ThermoFluor" denaturation have more significant slopes than with many other denaturation methods due to the temperature dependence of ANS fluorescence. Tm is determined, as usual, from the midpoint of the unfolding transition.

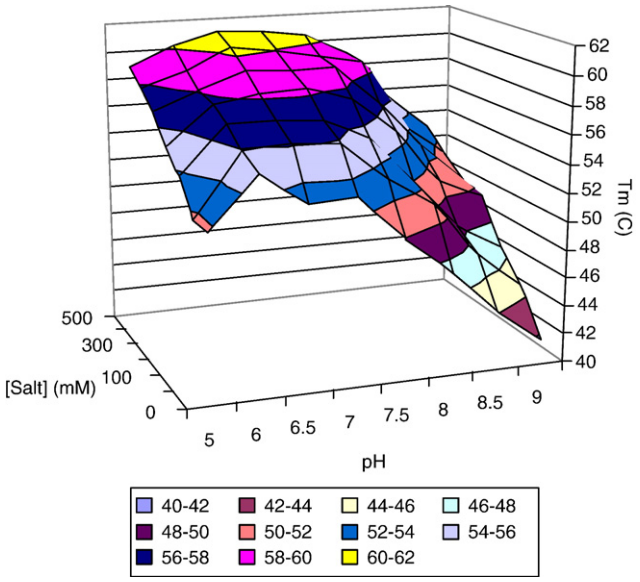

Fig. 2. Thermal stability landscape for Klenow DNA polymerase as a function of pH and salt concentration. The different colors in the graph represent different Tm values in 3 °C intervals from 40 to 62 °C. Klenow stability increases with increasing salt concentration and for the most part with decreasing pH. Tm's at pH's 5–6.5 and 7.5–9 are averages of duplicates in the same buffer. Each pH 7 Tm is an average of the Tm for Klenow at the specified salt concentration in four different buffers (sodium phosphate, PIPES, HEPES, and MOPS).

Simultaneous variation of both salt concentration and pH provides a stability surface or landscape for the protein, such as that shown in Fig. 2 for Klenow. The pH by [NaCl] landscape in Fig. 2 is, of course, only one of many such stability landscapes that one might examine for the protein. The use of different salts, or the inclusion or absence of "typical" protein chemistry additives (e.g. DTT, BME, EDTA, glycerol, surfactants, etc.) will potentially reveal different landscapes, or perhaps more correctly: a different transect through the global, multi-dimensional stability landscape for the protein. Furthermore, while we have focused only on Tm shifts, due to the irreversible denaturation of Klenow, stability landscapes for ΔG, ΔH, and ΔS versus solution conditions also exist and will be experimentally accessible for some proteins.

Fig. 2 shows that increasing the concentration of NaCl at any pH will stabilize the protein. The magnitude of the stabilization changes at different pH's, giving rise to the topology of the stability surface. Likewise, decreasing pH at any salt concentration stabilizes the protein, but unlike the case for salt, there is a clear but subtle maximum in the pH stabilization, which gives the landscape its slightly peaked or hill-like appearance. Depending on the salt concentration, the pH of maximal stability hovers around pH 6.5–7.5. The decrease in stability in the pH 7 to 5 range is slight compared to the dramatic increases in stability in the pH 9 to 7 range, and so much of our discussion focuses on the more dramatic proton stabilization effect, although it seems that an acid denaturation effect is likely starting to overlap at lower pH values. This onset of acid denaturation near pH 5.0 is also visible in Fig. 3.

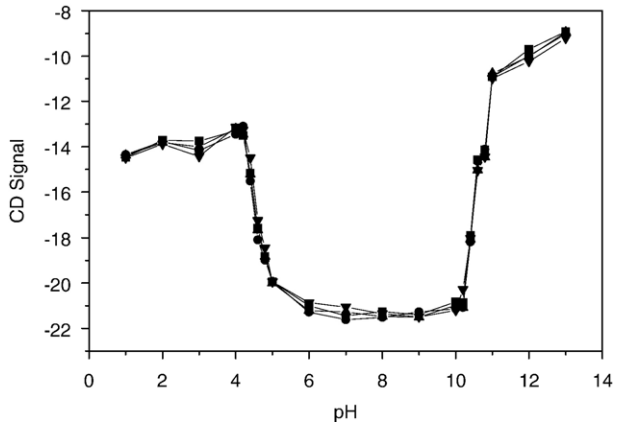

Fig. 3. CD spectroscopy of Klenow DNA polymerase as a function of pH. This figure shows the CD signal (millidegrees) for Klenow monitored at 221 (squares), 220 (circles), 219 (triangles), and 218 nm (inverted triangles), measured as a function of pH.

Fig. 3 is a plot of the circular dichroism of Klenow at 218–221 nm as a function of pH. In its native state, Klenow has a secondary structure trough in its CD spectrum in the 218–221 region, and this minimum is used to follow the thermal and/or chemical denaturation of the protein [21,22]. This figure illustrates the transition zones for acid and alkali denaturation of the protein, which have midpoints around pH 4.5 and 10.4, respectively. These data show that with the possible exception of experiments near pH 5, that the secondary structure of Klenow is in the native state throughout the pH range examined in Fig. 2. It is generally assumed that the protein is fully native under such conditions.

Examining the stabilizing effects of salts other than NaCl reveals several interesting features about the stabilization of Klenow, and these are illustrated by the data in Figs. 4–6. Fig. 4 shows the ΔTm for additions of different concentrations of chloride salts (NaCl, KCl, NH_4Cl, $MgCl_2$, and $MnCl_2$), sulfate salts (Li_2SO_4, $(NH_4)_2SO_4$, $MgSO_4$, and $NiSO_4$), and phosphate salts (ammonium and potassium phosphate), as well as two common divalent metal chelators EDTA and EGTA. The particular salt and concentration combinations used are those of a typical "salt and pH screen" currently conducted as part of the drug screening and discovery procedures at Johnson & Johnson Pharmaceutical Research and Development [26,27]. One can clearly observe both anionic and cationic effects in this figure. For example, for the four chloride salts of NH_4^+, Na^+, Li^+, and K^+, there is a clear hierarchy of cationic stabilization: $NH_4^+ > Na^+ > Li^+ > K^+$. Similarly, a comparison of sulfate or phosphate salts of the same cation, clearly show a 1–2° increase in Tm relative to chloride salts. The only salt that destabilizes Klenow is $NiSO_4$. In order to insure that the $NiSO_4$ destabilization

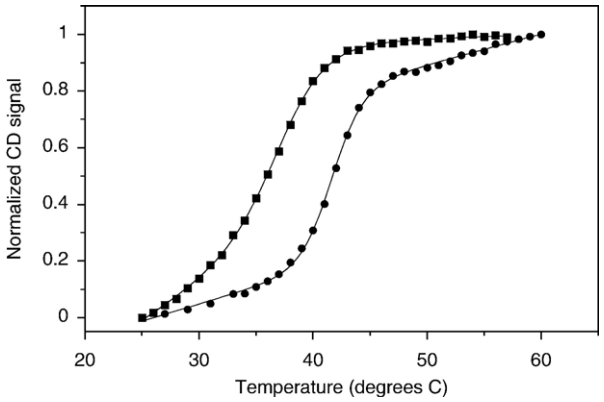

Fig. 5. Circular dichroism monitored thermal denaturation of Klenow polymerase in the presence (squares) and absence (circles) of 100 mM $NiSO_4$. Protein concentration was 0.05 mg/ml in 10 mM Tris, pH 7.9.

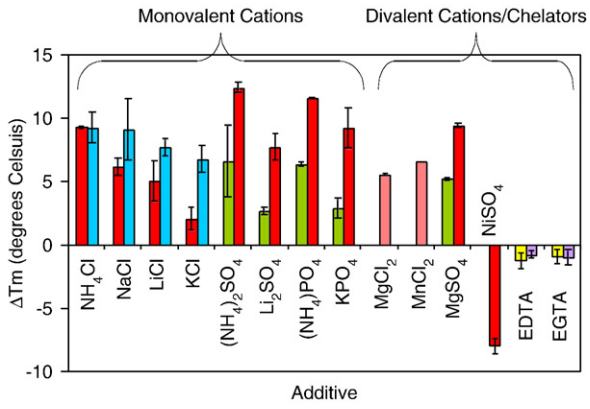

Fig. 4. Thermal stabilization of Klenow is dependent upon both salt type and concentration. Each bar in this graph represents the change in Tm (ΔTm) for Klenow in the presence of the indicated additive with respect to a reference Tm (53.2±0.5 °C). The reference Tm is an average of 16 Tm's for Klenow diluted into water instead of additive. Error bars are the standard deviations of duplicate measurements. The bar shading is indicative of the additive concentration. Salt concentrations are: 5 mM (pink), 10 mM (green), 100 mM (red), 300 mM (blue). Chelator concentrations are: 0.05 mM (yellow), 1 mM (purple).

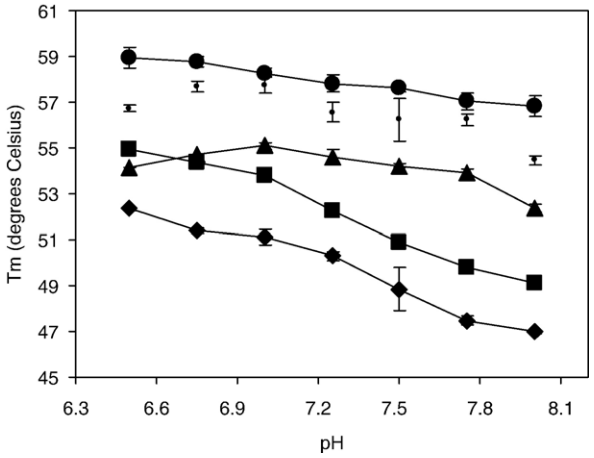

Fig. 6. NaCl and $MgCl_2$ each individually increase the thermal stability of Klenow, and the combined stabilizing effect of these two salts is synergistic. The data sets show the dependence of Tm on pH for Klenow in 25 mM HEPES buffer with no added salt (filled diamonds), 100 mM NaCl (filled squares), 5 mM $MgCl_2$ (filled triangles), or 100 mM NaCl and 5 mM $MgCl_2$ (filled circles). Errors on all filled symbols are the standard deviations of two independent Tm measurements. The small dots represent the calculated additive stabilization of NaCl and $MgCl_2$, along with the associated propagated error.

observed in the ThermoFluor assay was not related to some peculiarity of the assay, we also thermally denatured Klenow in the presence of $NiSO_4$ in a circular dichroism monitored assay. Fig. 5 thus confirms, using a more conventional assay, that the addition of $NiSO_4$ substantially lowers the Tm for Klenow.

Fig. 6 shows another two-dimensional transect through the stability data for Klenow, the pH vs. Tm profile for Klenow under four specific combinations of NaCl and $MgCl_2$ (or their absence). Under all four conditions, increasing pH decreases the Tm, if all the data are linearly fit. The pH effect in the presence of 5 mM $MgCl_2$, however, shows definite fine structure indicating there is a gentle (~1 °C) maximum in these data. It is notable, however, that across most of the pH range adding 5 mM $MgCl_2$ has a significantly larger stabilizing effect than adding 100 mM NaCl. This again reinforces the presence of a significant cationic stabilization effect (since there is only 10 mM Cl^- in 5 mM $MgCl_2$). It has long been known that Klenow binds Mg^{+2} (and Mn^{+2}) ions in its polymerization and exonuclease active sites [32,33]. These Mg^{+2} ions are tightly bound, however, with Kd values in the micromolar concentration range [32,33]. Even treatment of Klenow with 10 mM EDTA does not remove the Mg^{+2} ions necessary for DNA binding [30]. Nonetheless, the Mg^{+2} effect on Klenow stability may involve some overlap between specific active site bound Mg^{+2} uptake, and general surface Mg^{+2} association. Fig. 6 also suggests that the effects of Mg^{+2} and Na^+ are synergistic. Across the entire pH range, but most significantly at high and low pH, the measured Tm in the presence of both salts is slightly higher than the sum of the measured ΔTm's for the two individual salts.

4. Discussion

In the context of a mutational characterization of the nucleotide binding site, Joyce and associates measured Tm values for Klenow and several site directed mutants of Klenow using CD spectroscopy in a solution of 20 mM potassium phosphate, 15 mM NaCl, and 15% glycerol at pH 7.0 [23]. Under these conditions, a Tm of 55 °C was reported for wildtype Klenow [23]. These results were at odds with our own previous CD and calorimetric thermal denaturations of Klenow, which were conducted at high pH (9.5) and in the absence of salt in order to be directly comparable to parallel denaturations of Klentaq polymerase [21]. Under these no salt, high pH conditions we obtained a Tm of 37 °C for Klenow, and demonstrated that decreasing the pH to 7.5 (still in the absence of salt) raised the Tm to 47 °C [21]. The pH-salt stability landscape for Klenow determined in this study resolves the conflict between these previous measurements, and show that both Joyce's earlier Tm measurements and our own are easily explained by the pH-salt stability landscape for Klenow. Glycerol will also contribute somewhat to the previously observed differences in Tm for Klenow. We only examined Klenow in the presence of 1% and 5% glycerol (data not shown), and found that these additions caused 0.7 and 1.6 °C increases in Tm, respectively.

4.1. Anionic and cationic stabilization

The typically stabilizing, and occasionally destabilizing, effects of salt on proteins has a long history of investigation [1–14]. Several molecular mechanisms have been proposed to explain the effects of salt on protein structure, including preferential hydration, specific ion binding, and electrostatic screening of repulsive surface charge interactions. Stabilizing and destabilizing effects of ions are most often attributed to the anion, and the Hofmeister series has long been known to be a general predictive/correlative guide for the stabilizing versus destabilizing effects of particular anions on protein structure, although a few exceptions exist. Chloride is consistently in the "middle" of the Hofmeister series, where the stabilizing and destabilizing effects are believed to be largely balanced, while sulfate and phosphate are consistently listed as two of the most stabilizing anions (although their exact order in the series sometimes differ from publication to publication). Fig. 4 thus demonstrates clear Hofmeister effects on the stability of Klenow, since the sulfate and phosphate salts of any cation consistently show a 1–2 °C stabilization relative to the chloride salt for that cation.

Anions are frequently found to be primarily responsible for the salt stabilization of proteins [1,2,6,11]. Cation induced stabilization has also been observed, just less frequently [4,10]. Fig. 4 shows significant specific cation stabilization for Klenow polymerase. In chloride salts, the cationic stabilizing hierarchy is $NH_4^+ > Na^+ > Li^+ > K^+$. Fewer cation pairs were examined for phosphate and sulfate, but the stabilizing order to those that were examined is the same: $NH_4^+ > Li^+$ for sulfate and $NH_4^+ > K^+$ for phosphate.

4.2. Electrostatic surface topology

Previous electrostatic surface potential calculations performed for Klenow have shown that outside the positively charged DNA binding crevice, the majority of the surface of the polymerase has a net negative charge [28]. The electrotatic surface of Klenow is shown in Fig. 7. At physiological pH the protein will have a net negative charge of −7 to −8 (assuming each histidine residue contributes approximately +0.5 at neutral pH). Thus, the empirical stability behavior documented in this study can be well described by a model proposing that cations stabilize the protein by reducing the intrinsic repulsion within the predominantly anionic surface of the protein. Anionic dampening of surface cationic repulsion has been extensively studied by Fink and associates [2,11,14]. A similar mechanism for cationic stabilization, however, while previously hypothesized to be just as likely to exist [2], has proven somewhat empirically elusive [4]. Fink and associates have postulated that proteins that are stabilized by anionic attenuation of surface charge will tend to be proteins that are already marginally stable due to the surface cationic repulsion effects [2]. Klenow appears to follow this same model: we have previously shown that Klenow is very marginally stable for a protein of its size (68 kDa) [21,22].

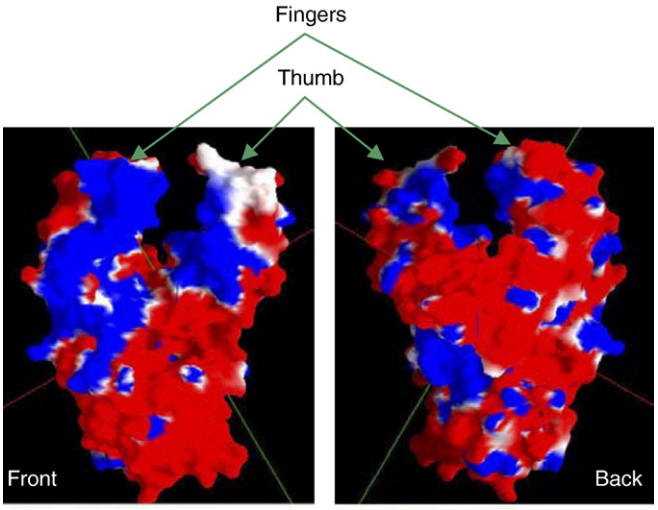

Fig. 7. Topology of the electrostatic surface potential for Klenow DNA polymerase. Patches of positive (+) and negative (−) potential are shown, respectively, in blue and red. Neutral patches are white. The electrostatic topology of Klenow was originally calculated and published by Warwicker and associates [26]. The "fingers" and "thumb" domains of Klenow DNA polymerase are labeled in these diagrams to orient the viewer.

4.3. Destabilization by nickel sulfate

Figs. 4 and 5 show an unusual, large destabilization of Klenow in the presence of $NiSO_4$. $NiSO_4$ destabilization of a number of proteins has been observed during the course of ThermoFluor drug screening at Johnson & Johnson Pharmaceutical Research and Development (data not shown). Since the vast majority of the proteins examined by ThermoFluor in this R&D program are his-tagged, it was generally assumed that the commonly observed $NiSO_4$ destabilization might originate from a preferential Ni^{+2}–histidine interaction in the denatured state, however the Klenow utilized in this study is not his-tagged. Ni^{+2} has been shown to generally stabilize human serum albumin and GroEL, and to specifically stabilize a variety of known Ni^{+2} binding proteins [34–37]. However, there is no evident prior documentation of a Ni^{+2} destabilizing effects on proteins, thus this large $NiSO_4$ destabilization effect may warrant further investigation. Our own preliminary examination of the effects of $NiSO_4$ on two other proteins found that this salt also destabilizes the adipocyte lipid binding protein, but stabilizes lysozyme (data not shown). However, the extremely low solubility of nickel salts in many biological buffer systems seriously impedes further examination of these nickel effects.

4.4. Mg^{+2} versus Na^{+} effects

Fig. 6 shows the Tm values for Klenow (1) in the absence of added salt, (2) in the presence of 100 mM NaCl, (3) in the presence of 5 mM $MgCl_2$, and (4) in the presence of 100 mM NaCl + 5 mM $MgCl_2$. At all pH's except 6.5, 5 mM $MgCl_2$ has a larger stabilizing effect than 100 mM NaCl. If stabilization primarily occurs via ion interaction with the protein, then the affinity of Mg^{+2} for the protein is at least 10 fold higher than the affinity of Na^{+} for the protein. As discussed above (in Results) this may reflect some tight binding of Mg^{+2} to the active sites of the polymerase [32,33]. The observation that combined Mg^{+2} and Na^{+} synergistically stabilize the protein to higher temperatures than the sum of their individual effects is also suggested by the data in Fig. 6. Such synergy would require that Mg^{+2} and Na^{+} binding sites interact with each other, and act cooperatively to enhance the stability of the protein. For example, one might envision protein associated Mg^{+2} and Na^{+} ions being arranged relative to the protein surface in such a way that they might be bridged by Cl^{-} or water, leading to a synergistic stabilization. Such anion or water bridging could create a dynamic, semi-continuous, weakly bound ionic veil over the protein anionic patches.

There are several major caveats to the conceptual molecular models postulated in this discussion. The first being that Tm need not necessarily be linearly additive like an enthalpy or free energy would be. Tm is a function of ΔG, ΔH, and ΔCp, but because the relationship is non-linear, perfectly linear combinations of ΔG's, ΔH's and ΔCp's could produce non-additive shifts in Tm values. Second, the irreversibility of unfolding means there will be some variability of Tm with rate of heating, but our previous studies have shown that this variability is minor compared to the pH and salt induced changes characterized herein [21]. Further, the models described postulate that the cations interact directly with the protein, rather than exerting their effects indirectly, such as via preferential hydration effects [12,13,38]. Finally, we have assumed that the observed pH and salt effects are primarily a reflection of native state interactions, versus denatured state interactions. Quite simply, it is the striking correlation between anionic surface patching and the relatively uncommon cationic stabilization that makes cationic shielding of surface charge repulsion seem a particularly attractive explanatory model for the empirical pH-salt thermal stability landscape of Klenow.

Acknowledgements

This work was supported by NSF Grant 0416568 (to VJL). AJR was supported by an NSF-IGERT Training Fellowship 9987603. We thank Catherine Joyce for the gift of expression clones and bacterial hosts for Klenow. We thank J&J PRD for hosting Allison Richard for an internship. We thank Daniel Deredge for help with Fig. 7.

References

[1] S. Muzammil, Y. Kumar, S. Tayyab, Anion-induced stabilization of human serum albumin prevents the formation of intermediate during urea denaturation, Proteins 40 (2000) 29–38.
[2] C. Nishimura, V.N. Uversky, A.L. Fink, Effects of salts on the stability and folding of staphylococcal nuclease, Biochemistry 40 (2001) 2113–2128.
[3] A.C. Apetri, W.K. Surewicz, Atypical effects of salts on the thermodynamic stability of human prion protein, J. Biol. Chem. 278 (2003) 22187–22192.
[4] B.K. Jha, N. Mitra, R. Rana, A. Surolia, D.M. Salunke, K. Datta, pH and cation-induced thermodynamic stability of human hyaluronan binding protein 1 regulates its hyaluronan affinity, J. Biol. Chem. 279 (2004) 23061–23072.

[5] D.P. Kumar, A. Tiwari, R. Bhat, Effect of pH on the stability and structure of yeast hexokinase A, J. Biol. Chem. 279 (2004) 32093–32099.
[6] F.I. Rosell, M.R. Mauk, A.G. Mauk, pH- and metal ion-linked stability of the hemopexin–heme complex, Biochemistry 44 (2005) 1872–1879.
[7] P.H. von Hippel, T. Schleich, The effects of neutral salts on the structure and conformational stability of macromolecules in solution, in: S.N. Timasheff, G.D. Fasman (Eds.), Structure and Stability of Biological Macromolecules, Marcel Dekker, New York, 1969, pp. 417–574.
[8] K.D. Collins, M.W. Washabaugh, The Hofmeister effect and the behaviour of water at interfaces, Q. Rev. Biophys. 18 (1985) 323–422.
[9] J.A. Schellman, Selective binding and solvent denaturation, Biopolymers 26 (1987) 549–559.
[10] C.N. Pace, G.R. Grimsley, Ribonuclease T1 is stabilized by cation and anion binding, Biochemistry 27 (1988) 3242–3246.
[11] Y. Goto, L.J. Calciano, A.L. Fink, Acid induced folding of proteins, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 573–577.
[12] T. Arakawa, R. Bhat, S.N. Timasheff, Why preferential hydration does not always stabilize the native structure of globular proteins, Biochemistry 29 (1990) 1924–1931.
[13] S.N. Timasheff, Solvent stabilization of protein structure, Methods Mol. Biol. 40 (1995) 253–269.
[14] V.N. Uversky, D.J. Segel, S. Doniach, A.L. Fink, Association-induced folding of globular proteins, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 5480–5483.
[15] A. Kornberg, I.R. Lehman, M.J. Bessman, E.S. Simms, Enzymic synthesis of deoxyribonucleic acid, Biochem. Biophys. Acta 21 (1956) 197–198.
[16] I.R. Lehman, M.J. Bessman, E.S. Simms, A. Kornberg, Enzymatic synthesis of deoxyribonucleic acid I. Preparation of substrates and partial purification of an enzyme from *Escherichia coli*, J. Biol. Chem. 233 (1958) 163–170.
[17] H. Klenow, I. Henningsen, Selective elimination of the exonuclease activity of the deoxyribonucleic acid polymerase from *Escherichia coli* B by limited proteolysis, Proc. Natl. Acad. Sci. U. S. A. 65 (1970) 168–175.
[18] C.M. Joyce, N.D. Grindley, Construction of a plasmid that overproduces the large proteolytic fragment (Klenow fragment) of DNA polymerase I of *Escherichia coli*, Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 1830–1834.
[19] C.M. Joyce, T.A. Steitz, Function and structure relationships in DNA polymerases, Annu. Rev. Biochem. 63 (1994) 777–822.
[20] T.A. Steitz, DNA polymerases: structural diversity and common mechanisms, J. Biol. Chem. 274 (1999) 17395–17398.
[21] I. Karantzeni, C.R. Ruiz, C.C. Liu, V.J. LiCata, Comparative thermal denaturation of *Thermus aquaticus* and *Escherichia coli* type I DNA polymerases, Biochem. J. 374 (2003) 785–792.
[22] A.J. Schoeffler, A.M. Joubert, F. Peng, F. Khan, C.C. Liu, V.J. LiCata, Extreme free energy of stabilization of Taq DNA polymerase, Proteins 54 (2004) 616–621.
[23] M. Astatke, N.D.F. Grindley, C.M. Joyce, Deoxynucleoside triphosphate and pyrophosphate binding sites in the catalytically competent ternary complex for the polymerase reaction catalyzed by DNA polymerase I (Klenow fragment), J. Biol. Chem. 270 (1995) 1945–1954.
[24] R. Sterner, W. Liebl, Thermophilic adaptation of proteins, Crit. Rev. Biochem. Mol. Biol. 36 (2001) 39–106.
[25] C.A. Royer, Fluorescence spectroscopy, Methods Mol. Biol. 40 (1995) 65–89.
[26] M.W. Pantaoliano, E.C. Petrella, J.D. Kwasnoski, V.S. Lobanov, J. Myslik, E. Graf, T. Carver, E. Asel, B.A. Springer, P. Lane, F.R. Salemme, High-density miniaturized thermal shift assays as a general strategy for drug discovery, J. Biomol. Screen. 6 (2001) 429–440.
[27] D. Matulis, J.K. Kranz, F.R. Salemme, M.J. Todd, Thermodynamic stability of carbonic anhydrase: measurements of binding affinity and stoichiometry using ThermoFluor, Biochemistry 44 (2005) 5258–5266.
[28] J. Warwicker, D. Ollis, F.M. Richards, T.A. Steitz, Electrostatic field of the large fragment of *Escherichia coli* DNA polymerase I, J. Mol. Biol. 186 (1985) 645–649.
[29] C.M. Joyce, V. Derbyshire, Purification of *Escherichia coli* DNA polymerase I and Klenow fragment, Methods Enzymol. 262 (1995) 3–13.
[30] K. Datta, V.J. LiCata, Salt dependence of DNA binding by *Thermus aquaticus* and *Escherichia coli* DNA polymerases, J. Biol. Chem. 278 (2003) 5694–5701.
[31] A. Nicholls, K.A. Sharp, B. Honig, B. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons, Proteins 11 (1991) 281–296.
[32] J.P. Slater, I. Tamir, L.A. Loeb, A.S. Mildvan, The mechanism of *Escherichia coli* Deoxyribonucleic acid polymerase I. Magnetic resonance and kinetic studies of the role of metals, J. Biol. Chem. 247 (1972) 6784–6794.
[33] G.P. Mullen, E.H. Serpersu, L.J. Ferrin, L.A. Loeb, A.S. Mildvan, Metal binding to DNA polymerase I, its large fragment, and two 3′–5′-exonulcease mutants of the large fragment, J. Biol. Chem. 256 (1990) 14327–14334.
[34] Y.H. Lee, H.S. Won, M.H. Lee, B.J. Lee, Effects of salt and nickel ion on the conformational stability of *Bacillus pasteurii* UreE, FEBS Lett. 522 (2002) 135–140.
[35] A.A. Sabourry, Application of a new method for data analysis of isothermal titration calorimetry in the interaction between human serum albumin and Ni^{2+}, J. Chem. Thermodyn. 35 (2003) 1975–1981.
[36] G.C. Melkani, G. Zardenata, J.A. Mendoza, The ATPase activity of GroEL is supported at high temperatures by divalent cations that stabilize its structure, Biometals 16 (2003) 479–484.
[37] A.V. Dias, D.B. Zamble, Protease digestion analysis of *Escherichia coli* NikR: evidence for conformational stabilization with Ni(II), J. Biol. Inorg. Chem. 10 (2005) 605–612.
[38] T. Arakawa, S.N. Timasheff, Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding, Biochemistry 23 (1984) 5912–5923.