Salt Dependence of DNA binding by Thermus aquaticus and Escherichia coli DNA Polymerases*  

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DNA binding properties of the Type 1 DNA polymerases from Thermus aquaticus (Taq, Klenataq) and Escherichia coli (Klenow) have been examined as a function of [KCl] and [MgCl₂]. Full-length Taq and its Klenataq “large fragment” behave similarly in all assays. The two different species of polymerases bind DNA with submicromolar affinities in very different salt concentration ranges. Consequently, at similar [KCl] the binding of Klenow is ~ 3 kcal/mol (150×) tighter than that of Taq/Klenataq to the same DNA. Linkage analysis reveals a net release of 2–3 ions upon DNA binding of Taq/Klenataq and 4–5 ions upon binding of Klenow. DNA binding of Taq at a higher temperature (60°C) slightly decreases the ion release. Linkage analysis of binding versus [MgCl₂] reports the ultimate release of ~1Mg²⁺ ion upon complex formation. However, the MgCl₂ dependence for Klenow, but not Klenataq, shows two distinct phases. In 10mM EDTA, both polymerase species still bind DNA, but their binding affinity is significantly diminished, Klenow more than Klenataq. In summary, the two polymerase species, when binding to identical DNA, differ substantially in their sensitivity to the salt concentration range, bind with very different affinities when compared under similar conditions, release different numbers of ions upon binding, and differ in their interactions with divalent cations.

The large fragment domains of the Type 1 DNA polymerases from Escherichia coli (Klenow) and Thermus aquaticus (Klenataq) are remarkably similar in structure (see Fig. 1), despite the fact that Taq polymerase functions at temperatures 40–60° higher than E. coli Pol I/Klenow (1–5). A number of laboratories have been exploring the basis for the structural stability of extremophilic proteins (for a recent review, see Ref. 6). This study focuses primarily on the functional characteristics of this homologous mesophilic/thermophilic pair of polymerases in an effort to understand the functional similarities and differences between the two polymerases. Herein we focus on the initial functional step in DNA replication; that is, the binding of the polymerase to DNA and the dependence of DNA binding on salt.

Taq polymerase is a single chain polypeptide with a molecular mass of 94 kDa (1–4). Due to its use in PCR, Taq polymerase is one of the most important biotechnological reagents in use in the world today. The enzyme has both polymerase activity and 5’ nuclease activity and is a member of the Pol I polymerase family by virtue of its similarity to E. coli Pol I DNA polymerase (1–4). Like other thermophilic eubacterial DNA polymerases (5), but unlike E. coli Pol I, Taq polymerase lacks 3’ → 5’ exonuclease activity (so called “proofreading activity”) (1–4). Removal of the 5’ nuclease domain from full-length E. coli Pol I (103 kDa) produces the 68-kDa Klenow fragment (7). Removal of the same domain from Taq polymerase produces the 62.5-kDa Klenataq fragment (8).

The non-covalent driving forces that lead to stable protein-DNA complexes are strongly influenced by the solution environment (salt concentration and type, temperature, pH, etc.). As a result of this dependence on the solution conditions it is impossible to understand the forces that drive these interactions based solely on structural considerations. Rather, the functional properties (thermodynamics and kinetics) of these interactions must also be investigated as a function of solution conditions to understand the origins of the stability of the complexes. Models and mechanistic explanations of function in the Pol I polymerase family are often extrapolated from one family member to another. These extrapolations are well justified given the structural similarities among the family members as revealed by the extensive series of recent co-crystal structures of different family members (for review, see Refs. 9 and 10) as well as sequence similarities among the active sites (9). Assays of function and mutagenesis studies, however, often reveal subtle and not so subtle differences among the family members (e.g. Refs. 11 and 12), but directly comparative functional studies between different family members are relatively scarce. In this study, we have characterized the basic binding equilibria of full-length Taq, Klenataq, and Klenow polymerases to DNA using a fluorescence anisotropy assay. We have further characterized the KCl and MgCl₂ dependencies of DNA binding by the different polymerases and have quantitated the linked ion releases upon DNA binding for all three polymerases. The results show that these two species of polymerase, when binding to the same DNA, differ in their salt “sensitivities,” their intrinsic affinities, their linked ion releases, and their need for bound divalent cations.

EXPERIMENTAL PROCEDURES

Proteins—The proteins examined are full-length Taq DNA polymerase, the Klenataq fragment of Taq polymerase (8), and the D424A mutant of Klenow polymerase (13). The Klenow D424A mutant lacks 3’-exonuclease activity and is commonly referred to as “Klenow exo minus” (KF exo−). It is the predominant variant of Klenow polymerase used in the majority of functional studies of Klenow over the past decade. The Taq DNA polymerase gene was cloned, and the protein was expressed and purified in our laboratory. The clone of Klenataq was constructed by Wayne Barnes at Washington University (8) and was obtained from the American Type Culture Collection (Manassas, VA). The clone for D424A Klenow fragment was a gift from Catherine Joyce at Yale University.

The gene for Taq DNA polymerase was PCR-amplified using the strategy and primers described by Engelke (14). The amplified product

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1 The abbreviations used are: Pol I, polymerase I; ROX, rhodamine X.
was cloned into a pTrc99A expression plasmid (Amersham Biosciences) using EcoR1 and BamH1 restriction sites. The gene was then cut from this clone using Nco1 and SalI restriction sites, re-ligated into a pET-15b expression plasmid (Novagen), and transformed into the BL21(DE3) strain of *E. coli* for expression and purification of the protein. The correct *Taq* coding sequence of the resulting plasmid (pKD-Taq) was verified by DNA sequencing. The protein purification procedure for *Taq* was based largely on the procedure described by Barnes (15) for Klenow with a number of modifications, as follows. Cell pellets were solubilized in the lysis buffer (50 mM Tris-Cl, 10 mM MgCl₂, 50 mM dextrose, 250 mM KCl, pH 7.9) with lysozyme and heated to 75 °C for 1 h. Contaminating nucleic acids were then removed by polyethylene glycol precipitation in the presence of 250 mM KCl. The supernatant containing the protein was loaded over a Bio-Rex 70 ion-exchange column, pre-equilibrated with KTA buffer (20 mM Tris, 22 mM (NH₄)₂SO₄, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, pH 7.9) to remove the excess polyethylene glycol. The flow-through was then loaded over a heparin-Sepharose column in the same buffer, and *Taq* polymerase was eluted with a 22–270 mM (NH₄)₂SO₄ gradient. The *Taq* eluent was applied to a second Bio-Rex 70 column in KTA buffer, pH 8.8, and the flow-through contained the purified protein. Klenow polymerase was purified as published (15) with the following modifications: no surfactant was used during purification, the ammonium sulfate precipitation step was omitted, and a second Bio-Rex 70 column, at pH 4.8, and the flow-through contained the purified protein. Klenow polymerase was purified as published (15) with the following modifications: no surfactant was used during purification, and the ammonium sulfate precipitation step was omitted, and a second Bio-Rex 70 column, at pH 9.1, was added subsequent to the heparin column. The flow-through of the second Bio-Rex column contained the purified protein. Klenow exo minus (plasmid pXS106) was transformed into *E. coli* expression strain C3776 and grown and induced as described previously (15). The protein was purified as previously described for full-length Pol I (17) with omission of the DEAE-cellulose column. No surfactant was used during purification or storage of any of the polymerases. Protein concentration was measured using the Bradford method (18).

DNA—Stoichiometric and equilibrium DNA binding experiments were performed with the following primer-template sets: 13/20-mer, 5’-TGGAGCAAGGTCGAATCAG-3’ and 3’-AGGCTGACGCGAGGCTGTCGAAA-5’, and 63/70-mer, 5’-ATCGCAGGTGACGCTGCAGGTGTAACCGGAGTGTTCGATCACCAGTCCA-3’ and 3’-ATGCGTGCGATGTTTGGCCACTTGCACGAGGCTGTCGAAA-5’. The 13/20-mer primer template set used is the same as that used for kinetic studies of Klenow DNA binding by Benkovic and co-workers (19). The longer primer-template pair (63/70-mer) was designed for use at higher temperatures. The 63/70-mer uses the same putative binding region sequence as the 13/20-mer, with added “random” sequence selected from the same region of the M13 bacteriophage sequence. DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. Fluorescently labeled DNA was labeled at the 5’ end of the primer strand with rhodamine-X (ROX) and was purchased directly from Integrated DNA Technologies.

**Fluorescence Anisotropy Assay**—ROX-labeled DNA was titrated with increasing concentrations of protein, and binding was monitored using the anisotropy signal change as protein-DNA complex is formed (20, 21). Fluorescence anisotropy measurements were performed using a FluoroMax-2 fluorometer equipped with an automated polarizer and regulated at the indicated temperatures. The excitation and emission wavelengths were 583 and 605 nm respectively, with 8-nm band-pass and an integration time of 10 s. For all the equilibrium titrations the DNA concentration used was 1 nM. In all the experiments the protein was titrated into fluorescently labeled DNA, with the total [DNA] < *Kₐ*. After each addition the sample was equilibrated at the required temperature for 8 min, and anisotropy was measured. All titrations were performed in 10 mM Tris, pH 7.9, buffer at the indicated salt KCl and MgCl₂ concentrations and temperature. The pH was adjusted by mixing Tris base and Tris-HCl. KCl and MgCl₂ concentrations were varied across the widest possible range for each polymerase. Limiting titrations at low KCl concentrations were those where the *Kₐ* approached the total [DNA]. Limiting values at high KCl concentrations were the point where well behaved, reproducible isotherms could no longer be obtained. For assays examining the effects of EDTA on binding, the titrations were carried out in 10 mM Tris, 10 mM EDTA, pH 7.9, with KCl concentrations of 50 mM for *Taq* and 300 mM for Klenow. Before measurement of the binding in the presence of EDTA the proteins were extensively dialyzed against the same buffer. Equilibrium binding curves were fit to both the equation above and to a simplified standard single site isotherm usable when the [DNA] is the following.

\[
\Delta \Lambda = \Delta \Lambda _T/2D_T \left( E_T + D_T + K_d \right) - \left( E_T + D_T + K_d \right)^2 - 4 \left( E_T, D_T \right)^2
\]  

(Eq. 1)

where \( \Delta \Lambda \) is the change in anisotropy, \( \Delta \Lambda _T \) is the total anisotropy change, \( E_T \) is the total polymerase concentration at each point in the titration, \( D_T \) is the total DNA concentration, and \( K_d \) is the dissociation constant (20, 22). When used to analyze stoichiometric binding curves, both \( D_T \) and \( K_d \) are allowed to vary during the non-linear regression, and the binding stoichiometry was determined as the ratio of the fitted \( D_T \) and the known \( K_d \). Equilibrium binding curves were fit to both the equation above and to a simplified standard single site isotherm usable when the [DNA] << \( K_d \), and which thus assumes effective equality between the free polymerase concentration and the total polymerase concentration,

\[
\Delta \Lambda = \left( \Delta \Lambda _T/E_T/K_d \right)(1 + E_T/K_d)
\]  

(Eq. 2)

where \( \Delta \Lambda \) is the change in fluorescence anisotropy, \( \Delta \Lambda _T \) is the total change in anisotropy, \( E_T \) is the total polymerase concentration at each point in the titration, and \( K_d \) is the dissociation constant for polymerase-DNA binding. Both equations have strengths and weaknesses for fitting equilibrium binding data. The fitted \( K_d \) in Equation 1 is quite sensitive to the exact fixed total DNA concentration and any uncertainty in the determination of this concentration, whereas in Equation 2 the actual difference between the total and free polymerase concentrations propagates directly into the fitted \( K_d \). Fits to the titrations with the very tightest (lowest) \( K_d \) values in each salt series in this study gave ≤10% variation in the fitted \( K_d \) values using Equations 1 and 2. All other titrations in all salt series gave, within error, identical fitted \( K_d \) values with both equations. Values and errors reported in the tables are those returned from fits to Equation 1. All non-linear fitting was performed using the program KaleidaGraph (Synergy Software). Linked ion release upon binding of the polymerases to DNA was calculated using a basic linkage relationship (23–26), e.g. for KCl the relationship is the following.

\[
\left| \ln (1/K_d) \right| / \left| \ln (K_d) \right| = \Delta N_{\text{ion}} = \Delta n K^+ + \Delta n Cl^-
\]  

(Eq. 3)
RESULTS

To obtain adequate amounts of Taq polymerase to perform biophysical studies, we cloned the gene for Taq polymerase into a overexpressing pET vector (Novagen) using modification of the strategy and amplification primers originally described by Engelke et al. (14). There have been a number of previously published or patented descriptions of the purification of Taq DNA polymerase (1–4, 14, 15, 27, 28, 29–34). For routine use in PCR, full purification of the polymerase is not required, and the majority of the published or patented descriptions of Taq purification are aimed at producing Taq for use in PCR. Our procedures have been designed for producing Taq for use in biochemical and biophysical studies. As such, no additional tagged sequences (such as His tags) have been added to the polymerase. Also, no surfactants have been used in the purification, since their effects on the polymerase remain uncharacterized. In addition, we assayed for potential damaging effects of the ubiquitous 70 °C heat incubation step included in published and patented Taq purification procedures. Full purification of the polymerase without the heat step was performed; however, this “cold prep” polymerase behaved identically in all of our assays as the normal “heat-prepared” polymerase (data not shown), and so the heat incubation step was retained in the final purification protocol.

Binding Stoichiometry of the Polymerases—Stoichiometric titrations of each of the polymerases versus DNA were performed using fluorescence anisotropy, and the results are shown in Fig. 2 for Taq polymerase. Polymerases were titrated into ROX-labeled DNA at high concentrations of DNA ([DNA] \( \gg K_d \)). The anisotropy of the DNA increases as protein binds due to the decreasing rate of molecular rotation of the DNA in the complex. The stoichiometry of binding was determined by fitting the data to Equation 1 as described under “Experimental Procedures.” The ratio of bound protein to DNA at saturation is 0.97 for Taq, 1.04 for Klentaq, and 0.82–0.95 for Klenow to the 13/20-mer primer-template pair. The binding stoichiometry to the longer 63/70-mer primer-template pair was 1.1 for Taq and 1.15 for Klentaq. As a control, it was determined whether unlabeled DNA could effectively compete with fluorescently labeled DNA for binding to the polymerase. The second curve in the stoichiometric titration plots of Fig. 2 shows a stoichiometric titration of protein versus double the amount of DNA, where half the DNA is ROX-labeled, and half is not. Only the fluorescently labeled DNA is anisotropically visible, so the displacement and apparent doubling of the stoichiometric value for this second curve indicates approximately equally effective binding of both the labeled and unlabeled DNA to the polymerases. Unlabeled DNA effectively competed with ROX-labeled DNA binding to all three polymerases. It should be noted that DNA labeled with fluorescein, which was examined first, is not effectively competed by unlabeled DNA for binding (data not shown), underscoring the importance of the unlabeled competitive control.

KCl Dependence of DNA Binding—Fig. 3 shows representative fluorescence anisotropc titrations of the binding of Klenow and Klentaq polymerases to identical pieces of DNA at several different KCl concentrations at 25 °C. Each titration curve fits well to a single site binding isotherm, and it can be seen from the precision of the data that even modest shifts in \( K_d \) can be readily quantitated. Fig. 4 shows the thermodynamic linkage plots for DNA binding as a function of KCl concentration (\( \Delta\ln K_d/\Delta \text{[salt]} \)) for the polymerases in the presence and absence of 5 mM MgCl\(_2\). The negative slopes of the linkage plots are indicative of net ion release upon formation of the protein-DNA complex (23–26). As observed for most DNA-binding proteins, DNA binding is linked to ion release for all the polymerases. Full-length Taq polymerase and the Klentaq fragment behave essentially identically. The exact linked ion releases are reported in Table I.

Several points are notable about these data. The most striking observation is the significant difference in salt ranges where sub-micromolar binding occurs. DNA binding across the ~10–300 nM \( K_d \) range occurs at KCl concentrations about an order of magnitude higher for Klenow than for Taq/Klentaq. This means that under similar solution conditions the binding of Klenow to DNA is much tighter than that of Taq/Klentaq. Both species of polymerase were titrated over the widest possible KCl concentration ranges that produced acceptable and...
analyzable titration curves. Although the two [KCl] ranges do
not overlap, by extrapolation the binding of Klenow is, on average, about 3 kcal/mol (−150 °C) tighter than the binding of Taq/Klenow to the same DNA at similar salt concentrations. This difference is also reflected in the extrapolation of the linkage plots to 1 M salt (ln [KCl] = 0), which provides an estimate of the non-electrostatic components of the binding interaction (24–26). At 1 M salt the binding free energy for Klenow to DNA is ≈ 2 kcal/mol tighter than the ΔG(binding)

of Taq/Klenow to the same DNA (Table I).

In addition to the intrinsic affinity difference between the E. coli and Taq polymerases, the slopes of the linkage plots with KCl indicate that the binding of Klenow to DNA results in the release of 1.5 more ions than the binding of Taq/Klenow to the same DNA both in the presence and absence of MgCl2. This is an ≈50% increase in the linked ion release for Klenow relative to Taq/Klenow. For both polymerases, removal of MgCl2 from the buffer increases the reported linked ion release by about 0.6 ions, indicating that part of the ion release in the presence of MgCl2 is due to magnesium ion release.

MgCl2 Dependence of DNA Binding—Binding titrations were also performed as a function of MgCl2 at fixed KCl concentrations for Klenow and Taq polymerases. Fig. 5 shows the individual titration curves at different MgCl2 concentrations for the two polymerases as well as the thermodynamic linkage plots for binding versus [MgCl2]. It is clear that binding of Klenow is linked to the release of Mg2+ across the entire [MgCl2] range, but the [MgCl2] dependence of Klenow DNA binding is not monotonic. The MgCl2 linkage plot for Klenow (Fig. 5C) indicates a linked Mg2+ release of 0.9 ions. For Klenow, on the other hand, the MgCl2 linkage plot and inspection of the titration curves themselves (Fig. 5B) both show the absence of a linked Mg2+ release (rather, a small uptake) upon binding of Klenow to DNA up until 10 mM MgCl2, whereas above 10 mM MgCl2 there is a net release of 1.2 Mg2+ upon binding (Table II).

To further investigate the Mg2+ requirements for DNA binding by the two polymerases, we EDTA-treated Klenaq and Klenow to remove all excess and weakly bound Mg2+ and then assayed DNA binding affinity in the presence of EDTA. In both cases, EDTA significantly decreases the affinity of the polymerases for DNA, as shown in Fig. 6. This effect is slightly more dramatic for Klenow than for Klenaq (13× reduction of affinity for Klenow versus a 5.5-fold reduction of affinity for Klenaq). Surprisingly, however, removal of EDTA almost completely restores the original binding affinity of the polymerases (data not shown). These data indicate that free Mg2+ is not required for DNA binding by either polymerase but suggest that some protein-bound divalent cations are required for highest affinity binding by both species of polymerase (see “Discussion”).

KCl Dependence of Taq Binding at High Temperature—T. aquaticus lives at a temperature optimum of 70–75 °C (35). Because it is possible that increased temperature may alter the way Taq polymerase binds DNA, we also assayed for salt dependence of binding at higher temperatures. Binding at 60 °C required design and use of a longer DNA primer-template pair (63/70-mer), since the shorter 13/20-mer primer-template had a Tm of −55 °C. Fig. 7 shows that the salt dependence of binding of Taq polymerase to DNA at the 2 temperatures is similar but slightly decreased at 60 °C relative to 25 °C. Kd values are reported in Table 3. Fig. 7 also shows the KCl dependence for the longer 63/70-mer DNA at 25 °C to account for any possible changes due to the differences between the two lengths of DNA. At 25 °C, the binding affinity of Taq for the 63/70-mer is somewhat tighter (−3–4 × or −1 kcal/mol) than the binding affinity for the 13/20-mer, but the ln1/Kd versus ln[KCl] dependencies are the same. It is also interesting to note that Taq binds the 63/70-mer DNA with somewhat higher affinity at 25 °C than at 60 °C. Thermophilic proteins are generally almost catalytically inactive at room temperature, and this is also the case for Taq, which has been shown to have little or no polymerization activity below about 40 °C (4). Taq and Klenaq bind DNA quite happily at 25 °C, however, and even lower. A complete characterization of the temperature dependencies of binding for these polymerases is currently in progress in our laboratory.

DISCUSSION

KCl Dependence of DNA Binding by the Polymerases—The linked ion releases for binding of the two different species of polymerases to DNA are both relatively small compared with the range of ion releases generally reported for DNA-binding proteins (24–26, 36, 37). Some similar small ion releases that have been reported include the release of 3 ions upon binding of Sso7d to DNA (38) and a 1–2 ion release upon binding of the α-subunit of E. coli RNA polymerase to DNA (39). Although the ion release is small for both species of polymerase, the binding of Klenow polymerase results in a larger release of ions than the binding of Klenaq or Taq polymerase. On the scale of the overall ion release for the two polymerases, this difference between Taq/Klenaq and Klenow is somewhat significant and is indicative of differences in the way the two polymerases interface with the DNA. Even though both species of polymerase bind to the same DNA with ≈ 1:1 stoichiometry, their different linked ion releases suggest that they bind with different footprints. Below, we discuss this in light of the available crystal structures of the binary complexes of the polymerases bound to primer-template DNA (40, 41).

The ion release for Taq polymerase at 60 °C is slightly lower than the ion release at 25 °C, further distancing the salt-dependent behaviors of the two species of polymerase when they are compared at temperatures near their respective optimal growth temperatures. It may be possible that the temperature dependence of the ion release for Taq is more complex than is revealed by looking at only these two temperatures and that we have fortuitously chosen two temperatures for Taq where the ion releases are similar. There may exist specific separate solution conditions under which Klenaq and Klenow polymerases release the same number of ions upon DNA binding, but under the identical solution conditions examined herein, they do not.

Table I also shows that the ln1/Kd versus ln[KCl] linkage plots for Taq and Klenaq have identical slopes. The Taq and
Salt Effects on DNA Binding by DNA Polymerases

**Table I**

**KCl dependence of DNA binding by Klentaq and Klenow with and without MgCl₂**

All titrations were performed at 25 °C in 10 mM Tris, pH 7.9, with the listed concentrations of KCl. Titrations performed with MgCl₂ contained 5 mM MgCl₂.

<table>
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<th>[KCl]</th>
<th>K_d with MgCl₂</th>
<th>Ions released*</th>
<th>ΔG at 1 u KCl</th>
<th>K_d without MgCl₂</th>
<th>Ions released*</th>
<th>ΔG at 1 u KCl</th>
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<td>401 ± 11</td>
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* Values determined from the slope of the ∆lnK_d versus ∆ln[KCl] linkage.

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Fig. 5. MgCl₂ dependence of equilibrium binding of Klentaq and Klenow DNA polymerases to 13/20-mer DNA (1 mM concentration in all titrations) monitored using fluorescence anisotropy at 25°C. A, equilibrium titrations of Klentaq in 10 mM Tris, 50 mM KCl, pH 7.9, at MgCl₂ concentrations of 2 mM (●), 3 mM (■), 5 mM (▲), 7 mM (☆), and 10 mM (○). B, equilibrium titrations of Klenow in 10 mM Tris, 300 mM KCl, pH 7.9, at MgCl₂ concentrations of 2 mM (●), 5 mM (□), 10 mM (▲), 15 mM (△), 20 mM (◇), 30 mM (×), 40 mM (■), and 50 mM (○). C, MgCl₂ linkages (∆lnK_d versus ∆ln[MgCl₂]) for DNA binding of Klentaq (■) and Klenow (○).

Klentaq ∆lnK_d versus ∆ln[KCl] dependencies, shown in Figs. 4 and 7, are nearly co-linear if plotted on the same plot. This finding suggests that in both cases only the polymerase domain of the protein is binding DNA, i.e. that the 5’-nuclease domain of full-length Taq does not bind DNA under these conditions. If both the 5’-nuclease domain and the polymerase domain of the full-length enzyme bound the DNA, one would expect the full-length protein to interact with a larger amount of the DNA and, hence, exhibit a different ion release.

**Intrinsic DNA Binding Affinity and Salt Tolerance Differences between the Polymerases**—One of the most striking differences between the DNA binding properties of Klenow versus Taq/Klentaq polymerases is that they differ in salt ranges for sub-micromolar binding. A consequence of this difference is that at any particular salt concentration, the relative affinity of Klenow polymerase for DNA is significantly tighter than that of Taq/Klentaq. This is further supported by the estimated difference in non-electrostatic binding free energies for the polymerases, estimated from the extrapolation of the KCl dependence data to 1 mM salt. Both of these lines of evidence indicate that Klenow binds DNA about 3 kcal/mol tighter than Taq/Klentaq DNA. The data at high temperatures for Taq polymerase show that this large affinity difference is not due to the fact that we are assaying the binding of Taq/Klentaq to DNA at 25 °C. This large difference in relative binding affinities for the two different species of polymerase, like the difference in linked ion release, further suggests differences in their initial DNA binding modes.

It should be noted that the expression clones for Taq versus Klenow polymerases in this study are from completely different sources, with no overlap of materials or design. The clone for full-length Taq was produced in our laboratory, whereas that for Klenow was produced by Wayne Barnes (8, 15). The fact that the clones from the two independent sources produce Taq and Klenow polymerase that behave almost identically indicates that the decreased DNA binding affinity of Taq/Klentaq is an intrinsic property of the polymerase and is not the result of some sort of unintentionally introduced mutation. Furthermore, we examined the possibility that the heating step, included in almost all published Taq/Klentaq purifications, might somehow have partially damaged the protein. However, Taq polymerase purified without the heating step behaves identically to the protein purified with the heating step.

It is currently not clear why the *E. coli* Klenow polymerase binds DNA with so much higher affinity than the equivalent polymerase from *T. aquaticus*. Another way to look at the data of Fig. 4 is to note that the two species of polymerase bind DNA with similar affinities in very different salt concentration ranges. However, neither *E. coli* nor *T. aquaticus* are halophilic. In fact neither will grow well in media containing >1% NaCl (42, 43). How sensitive their growth and viability are in the 0–1% salt range has not been characterized, however, so it
Titrations were performed at 25 °C in either 10 mM Tris, 50 mM KCl, pH 7.9 (Klentaq), or 10 mM Tris, 300 mM KCl, pH 7.9 (Klenow).

<table>
<thead>
<tr>
<th>[MgCl₂]</th>
<th>Klentaq K_d</th>
<th>Ions released*</th>
<th>[MgCl₂]</th>
<th>Klenow K_d</th>
<th>Ions released*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.7 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>12.8 ± 0.5</td>
<td>0</td>
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<tr>
<td>2</td>
<td>9.2 ± 0.3</td>
<td>0.9 ± 0.1</td>
<td>2</td>
<td>10.5 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>11.2 ± 0.3</td>
<td>5</td>
<td>5</td>
<td>8.5 ± 0.3</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>17.6 ± 0.5</td>
<td>10</td>
<td>10</td>
<td>8.1 ± 0.3*</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>23.5 ± 0.5</td>
<td>15</td>
<td>15</td>
<td>11.6 ± 0.2*</td>
<td>12 ± 0.14</td>
</tr>
<tr>
<td>20</td>
<td>38.7 ± 2</td>
<td>20</td>
<td>20</td>
<td>11.7 ± 0.5*</td>
<td>20</td>
</tr>
<tr>
<td>30</td>
<td>40</td>
<td>30</td>
<td>30</td>
<td>21.3 ± 0.7*</td>
<td>30</td>
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<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>37.4 ± 0.8*</td>
<td>40</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>52.4 ± 2*</td>
<td>50</td>
</tr>
</tbody>
</table>

* Values determined from the slope of the lnln/K_d versus ln[MgCl₂] linkage.

Values used to calculate linked ion release for Klenow.

is possible that there are subtle differences in the salt sensitivities of the two bacteria that might correlate with the salt sensitivities of their Pol I-type polymerases. Several other potential explanations for the observed differences may be postulated.

1) There may be other factors in the intracellular environments of the two bacteria that either lower the affinity of the E. coli enzyme or increase the affinity of the Taq enzyme in vivo or both (i.e. allosteric regulators, specific anions, other proteins, etc.). Specific anions have been shown to regulate DNA binding affinity in several systems (25, 26, 44).

2) High temperature stability of Taq/Klentaq may be achieved at the cost of the loss of high affinity DNA binding. 3) The two polymerases might show significant differences in sequence specificity, e.g. Taq might bind GC-rich sequences (more common in thermotolerant bacteria) tighter than mixed DNA sequences.

4) The operational or tolerable functional affinity range for Pol I-type polymerases may be quite wide in vivo, such that any affinity in the nanomolar to picomolar range is adequate for appropriate function or easily compensated for in vivo by increased/decreased expression of the particular polymerase. 5) These Pol I type polymerases from E. coli and T. aquaticus, although widely considered homologues, may not be completely functionally equivalent in the two bacteria, and therefore, might not be expected to have similar DNA affinities. 6) DNA may simply bind to the two polymerases in different ways. This point requires expansion. It has long been known that Taq DNA polymerase is devoid of 3′-exonuclease (or proofreading) activity. The proofreading activity of Klenow polymerase, on the other hand, is so efficient that it interferes with direct binding and kinetics studies, such that Klenow mutants with reduced or eliminated exonuclease activity have been used in almost all such studies for more than a decade. Removal of the exonuclease catalytic activity does not, however, necessarily abolish DNA binding to the exonuclease site. In fact the co-crystal of Klenow bound to primer-template DNA utilizes one of these exonuclease deficient mutants, yet unexpectedly shows the DNA bound to it in a so called “editing mode,” which includes contacts in the exonuclease domain, the edge of the polymerase domain cleft, and a second cleft that is formed upon DNA binding and runs roughly between the two active sites and roughly perpendicular to the polymerase domain cleft (40). Co-crystal structures of DNA bound to Klenow show the DNA bound further up into the polymerase active site cleft (although it does not pass all the way through the cleft) (41). The position of the DNA in the crystal structure of the binary complex of full-length Taq and DNA (45) is similar to that for Klentaq. The binary complexes for Klenow and Klentaq are the structures that are shown in Fig. 1. It may be that as 3′-exonuclease activity evolved, the strength of the interaction of the DNA with the exonuclease site shifted the location of the initial binding site for DNA.
Salt Effects on DNA Binding by DNA Polymerases

<table>
<thead>
<tr>
<th>[KCl] (mM)</th>
<th>$K_d$ (nM)</th>
<th>Ions released $^*$</th>
<th>$\Delta G$ at 1 s KCl (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding to 13/20-mer at 25 $^\circ$C</td>
<td>50</td>
<td>12.7 ± 0.5</td>
<td>2.8 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>39.7 ± 2.4</td>
<td>2.8 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>80.1 ± 3.3</td>
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</tr>
<tr>
<td></td>
<td>125</td>
<td>177 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>Binding to 63/70-mer at 25 $^\circ$C</td>
<td>50</td>
<td>2.8 ± 0.2</td>
<td>2.8 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>8.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>20 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>34 ± 1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>78 ± 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>175</td>
<td>83 ± 4</td>
<td></td>
</tr>
<tr>
<td>Binding to 63/70-mer at 60 $^\circ$C</td>
<td>50</td>
<td>12.1 ± 0.4</td>
<td>2.5 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>23.2 ± 1</td>
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<tr>
<td></td>
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<td>50.0 ± 3.4</td>
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<td>150</td>
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<tr>
<td></td>
<td>175</td>
<td>277.5 ± 19</td>
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</tr>
</tbody>
</table>

$^*$ Values determined from the slope of the $\ln K_d$ versus $\ln [KCl]$ linkage.

References
Salt Effects on DNA Binding by DNA Polymerases