Characterization of the 'glutamate effect' on the solution thermodynamics and function of the large fragments of the type I DNA polymerases from E.coli and T.aquaticus

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ABSTRACT

In this study, it is shown that the large fragments of the type I DNA polymerase from \textit{E.coli} (Klenow) and \textit{T.aquaticus} (Klentaq) display enhanced DNA binding affinity in glutamate vs. chloride. Across the relatively narrow salt concentration ranges often used to obtain salt linkage data, Klenow also displays an apparently decreased linked ion released ($\Delta n_{\text{ions}}$) in Kglutamate vs. KCl while Klentaq does not display such an effect.

The osmotic stress technique reveals that Klenow and Klentaq DNA binding is associated with the release of $\sim$500 to 600 waters in KCl. For both proteins, replacing chloride with glutamate results in a 70% reduction in the hydration change upon DNA binding (to $\sim$150-200), highlighting glutamate’s osmotic role.

To further examine this osmotic effect of glutamate, the salt-DNA binding linkages were extended up to 2.5 M Kglutamate. Consequently, a reversal of the salt linkage is observed above 800mM for both proteins. Salt addition titrations confirmed that rebinding of salt displaced polymerase to DNA occurs beyond 1M [Kglutamate]. Non linear analysis of the biphasic salt linkage indicates that the osmotic role of glutamate is responsible for the reversed linkage and allows the quantitative dissection of the ionic and osmotic behaviors. The similar effect of glutamate on the two polymerases results in a relatively constant affinity difference ($\Delta \Delta G_{\text{bind}}^0$) ($\approx$3kcal/mol) throughout the entire salt range.

The catalytic activity of both polymerases persists into higher [Kglutamate] than [KCl]. However, the re-association of the proteins on the DNA in high Kglutamate does not result in enhanced catalytic activity.
These data represent only the second documentation of an apparent reversed salt linkage for a protein-DNA interaction. This unusual behavior is quantitatively accounted for by a shifting balance of ionic and osmotic effects of the glutamate anion.
1.1 General Introduction to DNA Polymerase I

The accurate transmission and maintenance of genetic material is a central aspect of cell survival and prolongation of life. The continued transmission of genetic material from one generation to the next requires the ability to accurately duplicate the genome of an organism. This function is primarily performed by a family of enzymes called DNA polymerases that catalyze the synthesis of new DNA strands from a pre-existing template during the process of replication. Although a multitude of DNA polymerases from various organisms have been isolated and characterized, all DNA polymerases have been found to share some common traits. All DNA polymerases require the presence of 3’OH end of a primer terminus which is bound to a template strand of DNA containing the preexisting genetic information to be replicated. Based on Watson-Crick base pairing, the incoming deoxyribonucleotide (dNTP) is incorporated next to the 3’ OH of the primer terminus and across from the template base situated next to the primer-template junction. All DNA polymerases catalyze the polymerization reaction through the nucleophilic attack on the α-phosphate of the incoming dNTP by the 3’ OH of the primer terminus. The reaction is facilitated by a pair of divalent cations coordinated at the active site (Reviewed in ¹).

In eubacteria, there are five types of DNA polymerases (DNA polymerase I, II, III, IV and V). DNA polymerase I and III were identified as the two major replication enzymes whereas DNA polymerase II, IV and V are involved in the removal and repair of damaged DNA. DNA polymerase III is the main replication enzyme ². Using the 3’ OH of an RNA primer synthesized by primase, it performs the bulk of the synthesis of DNA by extending the leading and lagging strand in a highly processive manner. DNA polymerase I, a smaller and less processive polymerase, is mostly involved in the lagging strand synthesis. It is responsible for the removal
of ribonucleotides and subsequent replacement with deoxyribonucleotides of the RNA primers during the maturation of Okazaki fragments. Although DNA polymerase I is not responsible for the bulk of DNA synthesis, it is nevertheless the most abundant DNA polymerase in eubacteria with about 400 molecules per cell and plays a critical role in genome replication of bacteria\(^2\). In addition, early studies have also suggested that type I DNA polymerases are involved in DNA repair\(^3\). It is thought that DNA polymerase I could be involved in the displacement of damaged DNA and filling the resulting gap from the lesion\(^4;5\). DNA polymerase I was the first DNA polymerase isolated from *Escherichia coli* and characterized in 1955 by Arthur Kornberg\(^6;7;8\). The early and extensive study of DNA polymerase I from *E.coli* has made it a model DNA polymerase which is used as a basis for the understanding of other DNA polymerases. Since then, type I DNA polymerases from various organisms have been the focus of intense research and/or biotechnological use. One such DNA polymerase I is *Thermus aquaticus* DNA polymerase I (Taq polymerase). Due to its thermophilic properties, it has become one of the most important biotechnological tools as a result of its widespread use in the polymerase chain reaction (PCR)\(^9\). The research presented in this work will involve the study of the two proteins mentioned above: *E.coli* DNA polymerase I and *Taq* polymerase.

*Thermus aquaticus* is a eubacterium that was discovered and isolated in 1969 by T.D. Brock\(^10\). It was characterized to grow optimally at 70\(^\circ\)C to 75\(^\circ\)C. Accordingly, its molecular machinery was observed to maintain function at corresponding high temperature\(^11;12;13;14;15;16;17;18\). The origins of the thermostability and sustained functionality of proteins from such thermophilic organism also garnered intense research interest. While the differences between *E.coli* and *T.aquaticus* with respect to temperature are common knowledge, the two organisms also differ in their sensitivity to solution conditions. *T.aquaticus* was isolated from hot springs in Yellowstone National Park and is only found in freshwater conditions. It is highly sensitive to
changes in its environmental conditions such as salinity or pH. On the other hand, *E.coli* inhabits the GI tract of animals and is subject to a wider range of such environmental conditions. In vitro studies have long established that ionic components are disruptive to DNA binding as salt concentration is increased, therefore presenting a major challenge towards maintaining functional DNA binding for organisms which encounter environments with increased salinity. The control of the intracellular ionic composition and concentration allow some organisms to overcome such challenge. For instance, *E.coli* accumulates intracellular potassium glutamate as a response to heightened external salinity. Herein, I will present the characterization of the DNA binding properties of *E.coli* and *T.aquaticus* DNA polymerase I as a function of salt concentration, pH and osmotic stress. In particular, I will focus on the effect of potassium glutamate on DNA binding and attempt to dissect its thermodynamic origins. Furthermore, I will discuss the DNA binding properties of DNA polymerase I from *T.aquaticus* and *E.coli* in light of the respective organisms’ ability to sustain increases in salt concentration.

### 1.1.1/ The Homology of *E.coli* DNA Pol I and *T.aquaticus* Taq Polymerase

DNA polymerase I from *E.coli* and *T.aquaticus* are both single chain polypeptides with the ability to perform multiple enzymatic activities. They catalyze the elongation of a primer-template junction by adding incoming dNTPs to the 3’ end of the primer. Both enzymes also possess a 5’ nuclease catalytic activity. In addition to their ability to perform the same catalytic activity, *E.coli* DNA Pol I and *Taq* polymerase have been classified as homologs based on structural and sequence similarities. The two polypeptides have comparable size and molecular weights. *E.coli* Pol I is 923 amino acids long and 103 kDa while *Taq* polymerase is 832 amino acids long and 94 kDa. The domain composition of these enzymes is very similar. The 5’ nuclease domain is located at the N-terminus (residues 1-324 for *E.coli* Pol I and 1-291 for *Taq* polymerase) and is responsible for the 5’ nuclease activity. The polymerase domain is
located at the C-terminus (E.coli Pol I residues 520 to 928, Taq polymerase residues 424-832) with an intermediate proofreading domain (Pol I residues 324-519, Taq polymerase residues 292-493) \(^{22,23,24}\). The polymerase and proofreading domains are responsible for the faithful incorporation of dNTPs in the growing chain of DNA. Sequence alignments have revealed 39% sequence identity between the two full length polypeptides \(^{25,26}\) which increases to 49% for the polymerase domains \(^{25}\). Furthermore, sequence and structural alignments have also revealed the conservation of motifs which play central roles in the catalytic activities \(^{1,25,27,28}\). The two proteins differ in one significant aspect: the proofreading domain of Taq polymerase does not possess catalytic activity. Sequence and structural comparisons have determined that the proofreading domain displays the most differences between E.coli and T.aquaticus DNA polymerase \(^{1,25,27,28}\). Large stretches of deletions/insertions within the proofreading domain are responsible for most of the size difference between the two polypeptides. In consequence, several secondary structure elements are absent (See Figure 1). Furthermore, several highly conserved residues or sequence motifs necessary for exonuclease catalytic activities are absent or modified \(^{22,24,29}\). The lack of proofreading activity is responsible for the significant differences in fidelity of DNA replication between these two polymerases. Indeed, E.coli Pol I incorporates DNA with an error rate of 1 in 10^9 dNTPs incorporated \(^{30}\) while Taq polymerase misincorporates a dNTP every 10^5-10^6 incorporations \(^{31}\).

1.1.2/ The Large Fragments, Klenow and Klentaq

The partial proteolytic cleavage of E.coli Pol I \(^{23,32}\) yields two polypeptides which maintain their respective enzymatic activities. Of the resulting polypeptides, the large fragment was found to retain full-length polymerization activity. Further characterization of the large fragments, Klenow \(^{23}\) (68 kDa, from E.coli Pol I) and Klentaq \(^{33}\) (62 kDa, from Taq polymerase) showed that it was comprised of the polymerase and proofreading domain. The large fragments
have since been widely used for the biochemical and structural characterization of DNA polymerase I. In fact, the structure of the full length *E.coli* DNA Pol I has not been solved whereas several structures of Klenow both in the apo and DNA bound form have been reported. In contrast, the crystal structures of both full length *Taq* polymerase and Klentaq have been reported.

![Figure 1: X-ray crystal structure of Klenow (left, 1KFD) and Klentaq (right, 1KTQ) represented in ribbon diagram.](image)

Even though they display dramatically different thermostability, the crystallographic data revealed a high degree of similarity between Klenow and Klentaq in secondary and tertiary structure (Figure 1). Indeed, the global architecture of both proteins resembles the characteristic ‘open right hand’ structure common across all families of DNA polymerases (reviewed in 1; 25; 27; 28). It consists of a fingers subdomain, the thumb subdomain and the palm subdomain. The finger subdomain is involved in binding of the single stranded portion of the template beyond the
primer template junction and the binding of the incoming dNTP and subsequent presentation for catalysis. The thumb subdomain is involved in binding of the double stranded portion of the DNA. Finally, the palm subdomain contains the catalytic site. The secondary structures of Klenow and Klentaq are also highly similar. They are highly \( \alpha \)-helical in content and have highly conserved secondary structure with similar arrangement of secondary structural element. For instance, the only \( \beta \)-sheets structural elements are conserved at the base of the palm subdomain and at the base of the proofreading domain where the catalytic sites are found.

- **Critical Motifs and Residues Involved in the Catalytic Mechanism**

Co-crystal structures, mutagenesis and biochemical data have identified key regions which are critical to DNA binding and catalysis by the large fragments. There is a wealth of crystallographic data for Klentaq which includes binary, ternary complexes and multiple conformational states\(^{29,36}\). All co-crystals display Klentaq in a ‘polymerase mode’ with the 3’ primer terminus located at the active site of the polymerase domain consistent with the observation that the proofreading domain is inactive in \( Taq \) polymerase. On the other hand, only one co-crystal structure of Klenow in the presence of primed template DNA has been reported. Furthermore, the available Klenow co-crystal displays the ‘editing mode’ where the last four bases of the primer strand have been melted away from the template and the primer terminus is bound at the active site of the proofreading domain\(^{35}\) (Figure 2). However, biochemical studies revealed that ‘polymerase mode’ is significantly populated for Klenow in solution\(^{37,38}\). Together with the crystallographic data from Klentaq, biochemical and mutagenesis studies in Klenow\(^{39;40}\) have identified residues that are critical for DNA binding and catalysis of polymerization for both proteins. Structural and/or sequence alignment suggests that these residues are part of structural motifs that are highly conserved among the type I DNA polymerase family\(^{26,28}\), and
somewhat within other DNA polymerase families \(^1; 25; 26; 27\). These conserved motifs are located at the polymerase active site and are critical to the polymerization reaction.

The three motifs are called motif A, B, C \(^1; 25; 27; 28\). They participate in the binding of the divalent cations, the incoming nucleotide and its consequent presentation for catalysis. In Klenow and Klentaq, these motifs are highly similar and structurally superimposable \(^28\). Three carboxylate residues that are essential for catalysis are found within those motifs. In both proteins, it is thought that three conserved carboxylate residues (Asp 705, Asp 882 and Glu 883 for Klenow; Asp 610, Asp 785 and Glu 786 in Klentaq \(^26; 28; 36; 39; 40\)) coordinate two critical divalent metal ions for the catalysis of the nucleophilic attack of the \(\alpha\)-phosphate of the incoming dNTP by the 3’ OH of the primer terminus. One of the divalent ions is thought to promote the deprotonation of 3’ OH of the primer and stabilize the pentacovalent transition state of the \(\alpha\)-phosphate of the

Figure 2: X-ray co-crystal structure of Klenow (left, 1KLN\(^{,35}\)) and Klentaq (right, 4KTQ\(^{,36}\)) in ribbon diagram. The DNA is shown in wireframe representation.
incoming dNTP. The second divalent cation is thought to participate in the stabilization of the pentacovalent transition of the $\alpha$-phosphate and the departure of the pyrophosphate. It is generally thought that the divalent cations are $\text{Mg}^{2+}$, explaining the addition of magnesium in PCR. However, it is not clear if it is always the divalent cation co-factor used by these enzymes in vivo.

- **Critical Residues and Regions Involved in DNA Binding**

  Additional conserved regions have been identified within the type I polymerase family. Notably, a conserved region consisting of two helices and located at the tip of the thumb is critical for binding of the duplex portion of DNA. Upon binding, the thumb subdomain rotates towards the palm subdomain such that the tip of the thumb comes in close proximity and interacts with the sugar phosphate backbone of the minor groove. The deletion of this region was shown to have little effect on base substitution fidelity and catalytic activity but dramatic effects on DNA binding, processivity and frameshift fidelity of Klenow. The crystallographic data does not yield much information regarding the interactions of the protein with the single stranded potion beyond the primer-template junction, however photochemical cross-linking and mutagenesis have deciphered a path for the single stranded template on the fingers subdomain.

- **Inactive Proofreading Domain in Klentaq and Its Implication for DNA Binding**

  While the sequence, structural and functional data highlight the homology between the large fragments of *E.coli* Pol I and *Taq* polymerase, the two enzymatic activities and binding modes of Klenow present a significant functional difference between Klenow and Klentaq. The mechanism of proofreading activity, the competition between proofreading and polymerase activity and the differences in the binding modes have been studied. Crystal structures and mutagenesis have shown that the 3’ exonuclease activity performed by the proofreading domain is a nucleophilic reaction promoted by a two divalent metal ions mechanism. The two
divalent metal cations are coordinated by three carboxylate side chains (Asp 355, Glu 357, Asp 424 and Asp 501) \(^{43;45}\). These catalytic residues are found within three conserved motifs (exo I, II, and III) that are common to DNA polymerases that possess proofreading function\(^{46}\). These motifs and at least two of the catalytic residues are absent in Klentaq \(^{24;29;44}\). In Klenow, the mutation of one such residue (Asp 424) results in a mutant with dramatically decreased exonuclease catalytic activity but unaffected polymerase activity. However, the mutant retains the ability to bind in the ‘editing mode’ as illustrated by the co-crystal structure \(^{43;45}\). The factors involved in the determination of the mode of binding are currently the subject of much debate. However, it is generally believed that DNA binding mode switching occurs through the shuttling of the primer terminus between the two catalytic sites without dissociation of the DNA \(^{37;47;48}\).

In the polymerase mode, the primer template junction is located at the polymerase active site as observed in biochemical studies \(^{38}\) and in the co-crystal structures of Klentaq \(^{36}\). Upon partitioning to the 3’ exonuclease site located 30Å away, four bases located at the 3’ terminus of the primer are melted away from the template and shuttle to the proofreading domain amongst other possible conformational changes \(^{35}\). Biochemical studies suggest that in the absence of mispaired bases \(^{37;38}\) and divalent cations \(^{37}\), Klenow DNA binding is primarily in the polymerase mode.

**1.1.3/ The Kinetic Mechanism of Incorporation**

The kinetic mechanism for the incorporation of a nucleotide has been studied for Taq polymerase \(^{49}\) and *E.coli* DNA Pol I \(^{50;51;52}\). A consensus kinetic mechanism has been proposed for the incorporation of nucleotides by Type I DNA polymerases by Benkovic et al. \(^{53}\) (Figure 3). The proposed mechanism is composed of seven elementary steps, and multiple conformational states along the kinetic pathway have been corroborated by crystallographic data in Klentaq.
Figure 3: The proposed kinetic mechanism of nucleotide incorporation by DNA polymerase I on primed-template DNA. E is the enzyme, D$_n$ is the DNA with n nucleotide long primer, dNTP is in the incoming nucleotide and PPi is the leaving pyrophosphate. The altered conformational state associated with the slow non-chemical step is marked by an asterisk (*).
The first step is the formation of a binary complex upon protein-DNA binding. It is followed by the formation of the ternary complex with the binding of the incoming dNTP to the fingers subdomain. The crystal structures of both states have been reported for Klentaq. In addition, whereas the crystal structure of dNTP bound Klentaq is also available, the formation of a competent ternary complex requires the ordered binding of substrate on the protein. The third step is a slow non-chemical step that has been postulated to be a conformational change necessary for the nucleophilic attack to take place. The conformational change includes a significant movement of the fingers subdomain that leads to the formation of a closed ternary complex during which the incoming dNTP is presented to the polymerase active site. The crystal structures open and closed ternary complexes of Klentaq illustrate this conformational change. Furthermore, a second conformational change distinct from the fingers subdomain movement has also been proposed to take place during the third kinetic step. The changes have been postulated to be rearrangements of the active site geometry to allow the chemical step to occur and have been observed to be slower than the fingers movement. Furthermore, studies on misincorporation have suggested that these rearrangements may play a critical role in discriminating against the incorrect incoming nucleotide. The fourth kinetic step is the chemical step during which the 3’OH of the primer terminus performs a nucleophilic attack on the α-phosphate of the incoming dNTP promoted by the two divalent cations. Following the chemical step, another conformational rearrangement occurs which is followed by the release of pyrophosphate. Upon release of the pyrophosphate, the binary complex can either dissociate or translocate by a base and bind another nucleotide to enter another incorporation cycle in a processive manner. The full kinetic mechanism as proposed by Benkovic et al. is shown in Figure 3.
1.1.4/ Stability and DNA Binding of Klenow and Klentaq versus Temperature

DNA Pol I and Taq polymerase encounter significantly different physiological conditions resulting from the differences in the environmental habitats (temperature, salinity, pH) inhabited by these two organisms. Nevertheless, these two highly similar, homologous proteins carry out highly similar enzymatic reactions and fulfill similar functions for the two organisms. Therefore, it is essential to characterize the thermodynamic properties that govern the stability of the respective proteins and their association to DNA to understand the ability of E.coli Pol I and Taq polymerase to carry out similar functions in dramatically different conditions.

The basic thermodynamic characterization of the large fragments with regards to temperature has been performed. As anticipated from Taq polymerase’s thermostable properties and its use in PCR, Klentaq displays greater thermostability than Klenow. The melting temperatures (T_m) of the two proteins differ significantly with ~40-60°C for Klenow and 98°C for Klentaq 54. It shows that Klentaq maintains a native structure much further into high temperature than Klenow does. In addition, Klentaq also displays a ΔG of unfolding of 27 kcal/mol at 25°C which is significantly larger than Klenow’s ΔG of unfolding of 5 kcal/mol 55. The origins of the enhanced folding stability of Klentaq and other thermophilic proteins are still being studied. Nevertheless, these differences correlate well with the higher temperature tolerance of thermophilic T.aquaticus as opposed to the mesophilic E.coli.

Correlating with the difference in T_m, Klentaq is observed to maintain its DNA binding ability into much higher temperature than Klenow. The free energy of DNA binding to a primer template junction was found to vary nonlinearly between -9 and -10.5 kcal/mole for Klentaq in the range from 5°C to 70°C while Klenow’s varies nonlinearly between -10 to -11 kcal/mole in the temperature range between 5°C and 40°C 56; 57. However, the temperature of maximal DNA binding affinity was shifted higher in Klentaq (~45°C) than Klenow (~25°C). It highlights a
thermophilic adaptation which allows the Klentaq-DNA association to persist into higher temperature. Nonetheless, the thermodynamics of binding displayed similar $\Delta C_p$ values of 0.8 kcal/mol*K and 0.9 kcal/mol*K respectively. \(^56; 57\).

In a similar fashion, the thermodynamics of DNA binding of Klenow and Klentaq with regards to solution conditions such as salinity, pH and osmotic stress can be characterized and related to the respective organism’s sensitivity to such conditions. However, the semi-permeable cell membrane allows organisms to regulate their intracellular composition by acting like a barrier selecting out solution components which are deleterious to the cell and selecting for others. The characterization of the thermodynamics of the molecular machinery must take into account the intracellular variations of the respective organism’s response to variations in extracellular conditions.

1.2/ Osmoadaptive Behavior of \textit{E.coli} and \textit{T.aquaticus}

The major obstacle for an organism exposed to high salinity environments is the loss in turgor pressure. An increase in external salt concentration leads to an efflux of water, a loss of cytoplasmic volume and inhibition of cell division and proliferation. \(^58; 59\). Other cellular and metabolic activities such as respiration or transport can also be disrupted by the decrease in the intracellular water activity and the loss of cytoplasmic volume. \(^58\). Organisms that are adapted to higher salinity in their environment have evolved a mechanism to maintain turgor pressure and cytoplasmic volume. In some halophiles and extreme halophiles, it is achieved through the ‘salt-in-cytoplasm’ strategy in which the external inorganic ions are accumulated in the cytoplasm to match the external concentration with little effect on the metabolic activity. \(^59; 60\). On the other hand, most other organisms achieve osmoadaptation through the intracellular accumulation of compatible solutes to counteract the efflux of water. They are selectively imported or synthesized
intracellularly in order to restore turgor pressure with minimal disruption of cellular metabolic function \(^{59, 60}\).

_E.coli_’s response to increases in external salinity is well characterized. In minimal media, _E.coli_ can grow in conditions up to ~3\% NaCl (~1 Osm) \(^{61}\). The salt range in which _E.coli_ growth is observed can be extended to ~6\% NaCl (1M) or up to ~2 to 2.5 Osm through the addition of the compatible solute such as proline or glycine betaine \(^{62, 63, 64, 65}\). _E.coli_’s osmoadaptive abilities have been extensively studied and a general consensus has emerged from those studies. The primary response of _E.coli_ to the increase of external salinity is the import and accumulation of intracellular potassium ions, followed by intracellular accumulation of synthesized glutamate \(^{58, 59, 65, 66, 67, 68, 69, 70}\). First, potassium ions are imported by the _kdp_ transport system which is actively expressed in conditions of high osmolality or low turgor pressure \(^{58, 59}\). Then, the intracellular glutamate concentration increases. Neither the glutamate transport systems nor the synthetic pathways mediated by glutamate synthetase or glutamate dehydrogenase are osmoregulated \(^{59, 69}\). The accumulation of glutamate is thought to result from the inhibition of the macromolecular synthetic pathways that utilize the pool of glutamate following the osmotic shock and intracellular accumulation of potassium \(^{69}\). Along with the negative charges from the polyanionic nucleic acids, glutamate is the principal intracellular anion during osmoadaptation and it counterbalances the increase in positive charge caused by the increase in potassium ions. Indeed, Richey et al. first characterized that when the osmolality of the external medium was increased up to 1.1 osmolar, the intracellular potassium increased up to 0.93 molal while the intracellular glutamate increased up to 0.26 molal \(^{66}\). The accumulation of the two solutes allow the organism to counteract the changes in turgor pressure, the changes in membrane potential and the efflux of water caused by moderate changes in the salinity of the medium. In addition, several studies have suggested that the intracellular accumulation of
potassium glutamate plays a significant role in inducing further osmoadaptive strategies. The resulting increase in intracellular osmotic pressure causes a shift in gene expression to further the osmoadaptive ability of *E.coli*. The changes are conditional to the external medium composition. In the absence of compatible osmoprotective solutes in the media, the higher intracellular osmolarity caused by potassium glutamate induces the synthesis and accumulation of trehalose to further counteract changes in external osmolarity. In the presence of compatible osmoprotective solutes in the media, the increased intracellular osmolarity caused by potassium glutamate induces an increased expression of the osmoregulated proU and proP transport systems responsible for the import of such osmoprotectants as proline, betaine, proline betaine and glycine betaine. The accumulation of these solutes leads to a much improved osmoadaptation of *E.coli* as observed through the recovery of growth and/or respiratory activities.

Glutamate’s role in osmoadaptation is not restricted to *E.coli* and had been detected in the osmoadaptive response by many organisms across the bacterial and archeal domains. For instance, the archaeon *Methanococcus thermolithotrophicus* has been characterized as accumulating glutamate when grown in media with less than 1M NaCl. The accumulated compatible solute is switched to $N\varepsilon$-acetyl-$\beta$-lysine above 1M NaCl. Another such example is the hyperthermophilic archean *Palaeococcus ferrophilus*. More significantly, glutamate has also been implicated in the osmoadaptive response of halophiles. In one such case, glutamate was found to accumulate in the moderately halophilic *Halobacillus halophilus* at intermediate external salinities of ~1M (6% NaCl) and was involved in the switching of osmolyte strategies to proline for adaptation to higher salinities (12 to 18% NaCl).

In contrast to *E.coli*, little is known about the response of *T.aquaticus* to changes in environmental conditions. *T.aquaticus* is highly sensitive to external salt concentration which
corroborates the fact that it is found in fresh water environments. The growth of *T.aquaticus* in low concentration complex media (0.1% tryptone) is inhibited by 1 to 2% NaCl \(^{10; 83; 84}\). Furthermore, 1% tryptone was also found to be inhibitory. While there is little information on the intracellular content of *T.aquaticus, Thermus thermophilus*, a markedly more halotolerant member of the same genus was observed to accumulate trehalose and mannosylglycerate as the primary intracellular compatible solutes \(^{60; 84}\). The genes involved in the synthesis of these solutes were found, by southern blotting, to be absent in *T.aquaticus*. The same study found that a strain of *T.thermophilus* that lacked the ability to synthesize trehalose and/or mannosylglycerate displayed the same inhibition by NaCl concentration in the growth medium as that displayed by wild type *T.aquaticus* (1 to 2% NaCl) \(^{85}\).

**1.3/ The Effect of Salts on Macromolecular Processes**

**1.3.1/ The Enhancing Effect of the Physiologically Relevant Anion Glutamate**

The observed intracellular accumulation of potassium glutamate in *E.coli* in response to an external increase in NaCl prompted numerous studies on the consequences of substituting glutamate for chloride on macromolecular processes. For instance, glutamate has been found to increase the T\(_m\) of *E.coli* tubulin and enhance self-association into microtubules \(^{86}\). Glutamate also counteracts the urea unfolding of *E.coli* glutaminyl-tRNA synthetase \(^{87}\) and bacterial cell division protein FtsZ \(^{88}\) while promoting the polymerization of the latter into its functionally relevant tubule-like structures \(^{89}\). Furthermore, the presence of potassium glutamate was shown to have dramatic enhancing effects on *E.coli* expression systems from cell extracts or reconstituted from purified components. This effect was highly dependent on the identity of promoter and sigma factor which reflects the osmoregulated shift in gene expression when *E.coli* is subjected to osmotic pressure \(^{66; 72; 73; 74; 75; 90; 91; 92}\). In addition, substitution of glutamate for chloride has been described as enhancing the binding or the activity of individual *E.coli* DNA
binding proteins. Generally, the binding or optimal activity in glutamate salts is also extended and shifted to higher salt concentrations. Such systems include DNA polymerase III \(^93\), RNA polymerase \(^94\), various restriction enzymes \(^94\), Ribonuclease III \(^95\), T7 RNA polymerase \(^96\), transcription termination factor Rho \(^97\,98\), recA \(^99\), T4 coded gene 32 protein \(^99\), lac repressor \(^100\), ssb \(^101\) and IHF \(^102\).

1.3.2/ The Thermodynamic Study of Salt Effects on Protein-DNA Interaction

Understanding the origins of the dramatic anion specific effect of glutamate on protein-nucleic interactions requires thorough thermodynamic analysis of the effect of salt on protein DNA interaction. The framework for the thermodynamic study of salt effects on protein-DNA interaction was first established through the study of oligocations to DNA. Record et al. \(^19\) established that the net release of counterions from the vicinity of DNA is the driving thermodynamic force for binding of short olygo-lysine ligands to DNA and this explains the salt dependence of the interaction. They performed a thermodynamic ion linkage analysis which relates the decrease in equilibrium binding association constants to the concentration of salt. Under the assumption that only cations influenced the binding, a basic Wyman linkage predicts that the slope of the log-log plot \((d \log(K_a)/d \log[\text{Salt}])\) would yield the number of cations displaced from the vicinity of the DNA. In both single and double stranded DNA, the obtained parameter matched the theoretical number of cations per base condensed around DNA in the specific binding interface which is derived from the polyelectrolyte theory. These observations led to the conclusion that the net release of counterions from the vicinity of DNA was the major contributor to the thermodynamics of oligolysine-DNA interactions with regards to salt concentration. The ligand-DNA association results in a highly favorable entropic contribution from ion release at low salt concentration which grows less favorable as the salt concentration is increased. Under the assumption of absence of anionic and osmotic effects, this technique has led
to the experimental determination of the thermodynamically linked ion release upon protein-DNA interaction in order to estimate the number of protein-DNA electrostatic contacts. However, it was determined that the nature of the anion can modulate the thermodynamics of protein-DNA interactions and in particular the thermodynamically linked ion release parameter.

One such anion specific effect that garnered much interest was the physiologically relevant glutamate ion. In addition to increasing the salt concentration range at which binding is observed,
the substitution of chloride by glutamate significantly reduces the thermodynamically linked ion release in several DNA binding systems such as lac repressor, ssb, T4 gene 32 protein or IHF, further extending DNA binding to well beyond 500mM salt. Figure 4 shows the effect of substituting chloride by glutamate in some of the systems mentioned. These effects are not completely universal, however. For example, the α-subunit of RNA polymerase and the cAMP receptor protein do not display any anion specificity on binding or salt linkage at low salt concentration.<500mM>.

1.3.3/ The Thermodynamic Study of the Glutamate Effect

The interpretation of the anion specific behavior can be made in several ways. A first interpretation would assume that anion release contributes to the thermodynamic linkage of salt with protein-nucleic acids interaction. However, the anion specific effect on the linked ion release would result from difference in anion release. The different extents of anion release would result from differences in anion specific preferential exclusion from the vicinity of proteins. This interpretation is corroborated with the established observation that glutamate is more excluded from protein surfaces than chloride. In addition, recent studies on ssb and IHF have also reported a salt dependent enthalpy of DNA binding and in particular an anion specific salt dependence of the enthalpy of binding. In the presence of chloride, the enthalpy of binding is much more dependent on salt concentration than in glutamate or fluoride salt. This observation would corroborate the idea that anions contribute to the salt linkage and that anion specific effects would arise from anion specific interactions with the protein.

A second interpretation would involve the uptake of anions within the protein-DNA interface. One such case has been rigorously studied and reported. The TATA binding protein of the hyperthermophilic archon Pyrococcus woesei (PwTBP) displayed the unique and counter-intuitive behavior of increased equilibrium binding affinity as salt concentration is increased and
suggested the uptake of ions upon DNA binding $^{107}$. Additionally, the mutation of three basic residues on the surface of the protein led to the loss of the halophilic DNA-binding behavior and the display of more mesophilic behavior $^{108; 109}$. It is to be noted that halophile *P. woesei* accumulates potassium ions and di-myo-inositol-1,1’-phosphate in response to salinity $^{110}$. This unique behavior could be the result of drastic evolutionary adaptation necessary to maintain DNA binding in the presence of high intracellular salinity. Similarly, the anion specific behavior observed in *E. coli* DNA binding proteins could be the result of an anion specific moderate uptake of anion at the DNA binding interface.

Finally, the most thoroughly characterized studies (Lac repressor $^{100; 111}$, ssb $^{101; 112}$, IHF $^{102}$ and *PwTbp* $^{109}$) have also suggested that osmotic effects can contribute to the thermodynamic salt linkage of protein-nucleic acid interactions. Indeed, it is conceivable that ions that are relatively excluded from the macromolecular interface can result, upon DNA binding, in the displacement of osmotically stressed water which can greatly contribute to the thermodynamic salt linkage. While the aforementioned studies have attempted to incorporate the linked water release as a contributor to the thermodynamic salt linkage, the possibility of anion specific effect on the linked water release was not fully explored. As a consequence, the origin of such anion specific effects on the thermodynamics of protein-nucleic acid interactions and the nature of anion-protein interaction (in particular glutamate) remains largely unclear.

**1.3.4/ Preliminary Studies in Our System and Goals**

In our system, differences in DNA binding properties have previously been characterized with regards to solution conditions. Previous studies have characterized Klenow as much more salt resistant; binding of Klenow at a particular KCl concentration is 150 times (~3 kcal/mol) tighter than Klentaq binding to the same DNA. Similar submicromolar binding affinities were obtained for Klenow and Klentaq in the KCl ranges 250-500mM and 50-150mM respectively.
Salt linkage studies have determined that Klenow releases about 4.9 thermodynamically linked ions while Klentaq releases 3.4 ions in chloride salts with little dependence on the type of monovalent cation\textsuperscript{113}. This differential behavior with regards to salt concentration range correlates with the growth behavior of the respective organisms that was discussed in section 1.2.

The goals of this study are to determine how glutamate and other anions affect the thermodynamics of DNA binding by Klenow and Klentaq DNA polymerase and attempt to correlate these thermodynamics with the function of the proteins and the physiology of the respective organisms. These determinations would help us examine 1) the thermodynamic origins of the glutamate effect as observed in \textit{E.coli} macromolecular processes, 2) the potential molecular adaptations that have led to the respective thermodynamic and functional behavior of Klenow vs. Klentaq (and their respective organisms) with regards to solution conditions. For that purpose, we have characterized the linear thermodynamic linkages of the polymerase-DNA binding as a function of salt concentration and type, pH and water activity. The nucleotide incorporation of Klenow and Klentaq was also characterized as a function of salt concentration and specific anion type to examine the functional relevance of the observed binding behavior. Linear salt linkage analysis has revealed the characteristic glutamate effect on DNA binding in Klenow and somewhat in Klentaq. However, a dramatic effect of glutamate on the hydration change associated with both Klenow and Klentaq DNA binding was also revealed by the osmotic stress technique. This observation has led to the extension from the standard linear salt linkage analysis to a non-linear salt linkage analysis to characterize the combined salt and osmotic stress linkage. Such analysis required the examination of salt effects over a much wider concentration range than is typically examined. It has revealed that the glutamate effect predominantly results from a glutamate-induced osmotic effect which modulates inhibition versus enhancement of binding as potassium glutamate concentrations are increased. It has also revealed that Klenow
and Klentaq DNA binding displayed mostly similar behavior with respect to solution conditions and that the residual differences observed originate from a significantly higher intrinsic affinity of binding of Klenow relative to Klentaq. The effects of glutamate on DNA binding by both Klenow and Klentaq were also discussed in light of the functional behavior of the protein and the organismal behavior.
2.1 Preparation of Klenow and Klentaq Polymerases, DNA Oligonucleotides and Buffers

The proteins used in this study, Klenow and Klentaq are, respectively, the large fragments of *E.coli* DNA Pol I and *Taq* polymerase. The Klenow fragment used in this characterization is an exonuclease deficient D424A mutant \(^{45}\) (exo- Klenow fragment). The mutation removes an active site carboxylate involved in the coordination of the divalent cations critical for the exonuclease catalytic mechanism\(^ {45}\). The use of this mutant is critical in order to minimize the possibility of DNA degradation during DNA binding studies. Along with other exonuclease deficient mutants, the D424A mutant has been widely used for the biochemical and structural study of Klenow and *E.coli* DNA polymerase\(^ {35; 44; 114}\). The clone of the exo- Klenow fragment was kindly provided by Catherine Joyce at Yale University. The clone of Klentaq was constructed by Wayne Barnes and obtained from the American Type Culture Collections (ATCC)\(^ {33}\). The purification of Klenow and Klentaq\(^ {113}\) was based on published purification\(^ {33; 115; 116}\) procedures with slight modifications. For instance, all surfactants were removed from the purification procedures. The ammonium sulfate precipitation step in the published Klentaq purification procedure was replaced by a second Bio-Rex 70 column\(^ {113}\). For Klenow, the DEAE-cellulose column was omitted\(^ {113}\). Also, the type of salt used in the storage conditions was modified for Klenow. Potassium chloride was replaced by potassium glutamate following the observation in this dissertation work that Klenow’s DNA binding ability was maintained much longer when stored in glutamate-containing solutions rather than chloride. The protein concentrations were measured using optical density measurement at 280nm. The specific extinction coefficient of Klenow (0.86 (mg*mL\(^{-1}\))/O.D.) and Klentaq (1.13 (mg*mL\(^{-1}\))/O.D.) were determined using the Bradford method.
The primed-template DNA construct (13/20mer) used in the binding studies was the following:

\[
\begin{align*}
5' & \text{TGCAGCGTCCA} & 3' \\
3' & \text{AGCGTCGGCAGGTTCCAA} & 5'
\end{align*}
\]

The 13/20mer construct was the same as the one used by Benkovic et al.\textsuperscript{117} in the kinetic studies of Klenow-DNA binding. Rhodamine-X (ROX), a fluorescent probe was covalently attached to the 5’ end of the primer strand. The template strand and the fluorescently labeled primer strand were purchased from Integrated DNA Technologies (Coralville, Iowa). The primer and template strand were separately quantitated using the company provided molecular extinction coefficients at 260nm and then annealed in STE buffer (10mM tris.Cl, 1mM EDTA, 50mM NaCl, pH 8).

All chemicals used were reagent-grade and buffers were made using distilled, deionized water filtered through a US type I filter system. All assays (except circular dichroism) were performed in 10mM Tris adjusted for pH, salt concentration and osmolyte content. The pH was adjusted accordingly with the anionic salt (glutamic acid for potassium glutamate, HCl for KCl, HF for KF, aspartic acid for potassium aspartate and acetic acid for potassium acetate). The statistical fitting of all the resulting data was performed using KaleidaGraph from Synergy Software (Reading, Pennsylvania).

### 2.2 Experimental Procedures

#### 2.2.1 Fluorescence Anisotropy Titrations

Fluorescence anisotropy is a spectroscopic technique that monitors the loss of polarization of fluorescently emitted light\textsuperscript{118; 119}. The applicability of fluorescence anisotropy to study equilibrium protein-DNA interaction has been demonstrated in previous studies\textsuperscript{103; 118} including for Klenow and Klentaq\textsuperscript{56; 57; 113}. A fluorescence anisotropy assay was used to study the DNA binding properties of Klenow and Klentaq to the rhodamine-X labeled 13/20mer construct.
The emission and excitation wavelengths used were 583nm and 605 nm respectively. A polarizer along the excitation path is used to excite the fluorophore in the sample cuvette with linearly polarized light. The resulting fluorescent emission displays a loss of polarization as a result of rotational motions of the fluorophore and the DNA during the lifetime of the fluorophore. A polarizer placed in the emission path allows the measurement of the intensity of light emitted by the fluorophore which is polarized in parallel and perpendicular with respect to the excitation electromagnetic wave. The anisotropy (r) is obtained using the following relationship:

\[ r = \frac{(I_{VV} - I_{VH})}{(I_{VV} + 2I_{VH})} \]  

(Eq. 1)

With \( I_{VV} \) and \( I_{VH} \) representing the intensity of vertically and horizontally polarized light emitted by fluorophore following excitation with vertically polarized light. The anisotropic signal derived from those measurements is dependent on the motions experienced by the fluorophore.

Upon protein-DNA association, the difference in molecular weight and shape between the bound and free DNA results in a difference in the rotational motion experienced by the fluorophore. This difference in motion is directly reflected in the anisotropic signal obtained and therefore allows monitoring of the fractional saturation of the DNA with protein. In order to obtain reliable information, it is necessary to insure the absence of protein-fluorophore contacts which could induce changes in fluorescence anisotropy but do not reflect the DNA binding properties of the protein. The steady-state fluorescence emission spectra of the free and protein bound ROX-labeled 13/20mer did not show any significant differences which suggests that the environment of the fluorophore on the 13/20mer is not significantly altered upon binding by a protein. In addition, the steady-state fluorescence at 605nm does not change during the binding
titration again emphasizing that the protein does not appear to interact directly with the fluorophore, but alters its anisotropy through the interaction with the DNA.

The fractional saturation of DNA by Klenow or Klentaq was monitored by fluorescence anisotropy of the ROX-labeled 13/20mer in various assays. The fluorescence anisotropy assays were performed on a Fluoromax-2 (HORIBA Jobin Yvon, Edison, New Jersey) equipped with automated polarizers or an ISS Koala spectrofluorometer (Champaign, Illinois). All anisotropic assays were performed in quartz cuvettes. Titrations were performed by adding no more than a total of 1mL into an initial 2ml volume containing 1nM DNA. Following each addition, the sample was allowed to reach equilibrium for 8 minutes. The temperature inside the cuvette was maintained at 25°C using a circulating water bath and the sample was stirred to maintain homogeneity. Each data point consists of an average of 5 measurements using an 8nm band pass and a 10s integration time.

Fluorescence anisotropy was used to perform two types of assays: a forward equilibrium assay and a salt addition assay. Forward equilibrium titrations were performed by adding increasing amounts of protein into an initial solution which contained 1nM of fluorescently labeled DNA in the appropriately buffered solution conditions. The DNA was extensively diluted down to 1nM to obtain the initial 2mL solution. The protein was extensively dialyzed into the same buffer to make the titrant. In order to avoid dilution effects on the anisotropic signal, no more than 1mL of the titrant is added into the solution. For salt addition titrations, 1nM of ROX labeled DNA was saturated with Klenow (5nM) or Klentaq (500nM) at low salt concentration (5mM) in the initial 2mL solution. The salt concentration was then increased by titrating in high salt concentration containing buffers (4M KCl or 3M Kglutamate) while the anisotropic signal was monitored. In order to maintain the protein and DNA concentrations constant throughout the entire titration, the high salt titrant also contained the same concentration.
of ROX-labeled 13/20mer and protein as the initial solution. In addition, background titrations were performed to account for and subtract the effect of salt concentration and type on the anisotropic signal of the fluorescent DNA. These background titrations were obtained by replicating the titrations with the protein omitted from the initial solution.

### 2.2.2 Circular Dichroism

Circular dichroism (CD) was used to monitor the secondary structure of Klenow and Klentaq to investigate the possibility of salts and anion specific effects on protein stability that might be coupled to DNA binding. Circular dichroism is a spectroscopic technique that takes advantage of differences in absorbance of left vs. right circularly polarized far UV light by protein secondary structure elements. The loss of the secondary structure elements during unfolding can therefore be monitored by CD\textsuperscript{120, 121}. We have performed thermal unfolding of Klenow and Klentaq DNA polymerases (0.5mg/mL) in the presence and absence of various anionic salts (100mM KCl, KF and Kglutamate) using an Aviv 202 DS circular dichroism spectrophotometer (Aviv Biomedical, Lakewood, New Jersey). The unfolding was performed in 10mM phosphate buffer adjusted for pH and salt concentration. Glutamate was observed to be optically active in the far UV signal resulting in large errors and uncharacteristic CD signals. The use of an equal molar mixture of L and D isomers of glutamate helped stabilize the CD signal in the far UV region and restored the characteristic CD spectrum of Klenow and Klentaq. Other experiments in this dissertation demonstrate that the D and L isomers of glutamate have the same effect on the DNA binding of Klenow and Klentaq. The CD signal still had to be monitored at 227nm in the presence of glutamate, and 222nm for the other salt conditions. During thermal unfolding, the temperature was increased from 25°C to 70°C for Klenow and 80°C to 110°C for Klentaq. Temperature steps were minimal, 1°C, around the transition. The sample cuvette was sealed with a screw cap to avoid evaporation.
2.2.3 Nucleotide Incorporation

A nucleotide incorporation enzymatic assay was performed to determine the steady state rate and total amount of nucleotide incorporated by Klenow and Klentaq. The consequence of varying the salt concentration and anion type on these parameters were also assessed. The nucleotides incorporation assay and analysis was implemented in the LiCata laboratory by Hiromi Brown based on a published protocol\(^50\). The assay monitors the incorporation of radioactive \([\alpha^{32}\text{P}]\text{dATP}\) into primed M13mp18 DNA. The total volume of the reaction was 25uL and the reaction conditions were as follow:

- 10nM M13mp18 DNA primed with 63mer,
- 1uM protein (Klenow or Klentaq),
- 200uM of dCTP, dGTP and dTTP, and 150uM of dATP,
- 100uCi/mL of \([\alpha^{32}\text{P}]\text{dATP}\) (obtained from Perkin-Elmer, Waltham, Massachusetts).

The enzymatic reaction was carried out in a 10mM Tris, pH 7.9 buffer containing 0.1% Tween20 and the appropriate salt type and concentration. The reactions were performed at 35°C. The reaction was started by the addition of protein and incubated in a water bath. At various time points, the reaction was stopped by the addition of 25uL of 300mM EDTA. In KCl, the time points were 30, 60, 90 and 120 minutes. In Kglutamate, the time points were 20, 40 and 60 minutes. These time points were chosen within the steady state phase of the enzymatic reaction. Following the addition of EDTA, 10uL of the reaction was spotted on a DE81 filter obtained from Whatman (Piscataway, New Jersey). The spotted filters were then washed in a solution of 300mM sodium phosphate pH 7. Three consecutive washes of 5 minutes were performed followed by a final wash in 70% ethanol. The resulting filters were then dried and the radioactivity retained on the filter counted using a Tri-Carb 2900TR Liquid Scintillation Analyzer from Packard Bioscience (Perkin-Elmer). For the purpose of accurate determination
and analysis of the incorporation, background and total radioactivity measurements were performed. In the background measurements, the addition of protein was omitted. In the total radioactivity measurement, the wash steps were omitted and the spotted radioactivity including unincorporated radioactive dATP was directly measured.

The 7248 base long M13mp18(+) DNA was obtained from Amersham Pharmacia Biotech (GE Healthcare). The DNA was then transformed into competent cell line JM109 obtained from Promega. The isolation of M13 followed the published protocol\textsuperscript{122}. The M13 DNA was annealed with a single stranded 63mer displaying a complementary sequence to a specific region of the circular single stranded DNA. As a result, the M13 DNA displayed a primed template junction required for polymerization. The molecular weight of the primed M13 DNA was \~2.41MDa. The single stranded M13 was quantitated using the standard specific O.D. of single stranded at 260nm (30ng/uL per O.D.). The five bases immediately surrounding the primer template junction were identical in sequence as that of the primed template junction of 13/20mer fragment used for the DNA binding studies by fluorescence anisotropy.

2.3 Data Analysis

2.3.1 DNA Binding Studies by Fluorescence Anisotropy

2.3.1.1 Equilibrium Forward Titration

The anisotropic signal was used to monitor complex formation as protein was titrated into the sample cuvette containing 1nM ROX labeled 13/20mer in the appropriate solutions. Previous studies have established the 1 to 1 stochiometry of DNA binding by these two proteins\textsuperscript{113} to the ROX-labeled 13/20mer construct. Consequently, the anisotropic signal was normalized and fit to the following single site isotherm in order to estimate the equilibrium dissociation constant (K_d):

\[
\Delta A = \frac{\Delta A_r (E_r / K_r)}{(1 + E_r / K_r)}
\]  
(Eq. 2)
where $\Delta A$ is the change in anisotropy, $\Delta A_T$ is the total amplitude change in anisotropy and $E_T$ is the total protein concentration at each point. Equation 2 assumes equality between total protein concentration ($E_T$) and free protein concentration. In conditions yielding a $K_d > 10 \text{nM}$, such assumption is verified and the propagation of the difference in concentration of total and free protein into the fitted $K_d$ remains insignificant. In conditions yielding a $K_d < 10 \text{nM}$, the fractional saturation was also fitted to the following equation:

$$
\Delta A = \Delta A_T / 2D_T \left\{ (E_T + D_T + K_d) - \left[ (E_T + D_T + K_d)^2 - 4E_T D_T \right]^{1/2} \right\} \quad \text{(Eq. 3)}
$$

where $\Delta A$ is the change in anisotropy, $\Delta A_T$ is the total change in anisotropy, $E_T$ is the total protein concentration at each point, $D_T$ is the total DNA concentration and $K_d$ the dissociation constant. Equation 3 is more sensitive to the propagation of errors from the determination of the total concentration of DNA. In this dissertation, even the fits to the titrations with very low $K_d$ values ($< 10 \text{nM}$) resulted in less than 10% variation in the obtained $K_d$ using equations 2 and 3.

### 2.3.1.2 Linear Thermodynamic Linkage Analysis

**Salt linkage analysis** was performed by obtaining dissociation constants at various salt concentrations. The linear thermodynamic salt linkage analysis is similar to that originally applied by Record *et al.*\textsuperscript{19} to study DNA binding by oligolysines and then extended to protein-DNA interactions\textsuperscript{111; 112}. Using a basic Wyman linkage analysis\textsuperscript{124}, the number of thermodynamically linked ions released, $\Delta n_{\text{ions}}$ (anion and cation) and the $\Delta G^{\circ}_{1M}$ can be estimated by the slope and the intercept of the plot of $\ln(1/K_d)$ vs. $\ln[\text{salt}]$.

The reaction equation describing the protein (P)-DNA (D) interaction, taking into account the anions ($\Delta n_\text{A}$) and cations ($\Delta n_\text{C}$), is as follows:
\[ PA_{\Delta n_{\Delta_e}} + DC_{\Delta n_{e^+}} \leftrightarrow PD + \Delta n_{A^-} A^- + \Delta n_{C^+} C^+ \] (Eq. 4)

Equation 5 is the expression of the equilibrium protein-DNA association constant including the ions exchanged in the process:

\[
1 / K_d = \frac{[PD][A^-]^{\Delta n_{\Delta_e}} [C^+]^{\Delta n_{e^+}}}{[PA_{\Delta n_{\Delta_e}}][DC_{\Delta n_{e^+}}]} = \frac{[PD][Salt]^{\Delta n_{ions}}}{[PA_{\Delta n_{\Delta_e}}][DC_{\Delta n_{e^+}}]} \] (Eq. 5)

The logarithmic linearization of the expression of the equilibrium binding constant as a function of salt yields the linkage relationship:

\[
\ln(1 / K_d) = \ln\left(\frac{[PD][Salt]^{\Delta n_{ions}}}{[PA_{\Delta n_{\Delta_e}}][DC_{\Delta n_{e^+}}]}\right) = \ln([Salt]^{\Delta n_{ions}}) + \ln\left(\frac{[PD]}{[PA_{\Delta n_{\Delta_e}}][DC_{\Delta n_{e^+}}]}\right) = \Delta n_{ions} \ln(salt) + \ln(1 / K_d^{1M}) \] (Eq. 6)

Which is equivalent to:

\[ \Delta n_{ions} = \Delta n_{C^+} + \Delta n_{A^-} = \frac{d \ln(1 / K_d)}{d \ln[salt]} \] (Eq. 7)

The slope of the salt linkage yields the number of ions exchanged upon protein-DNA association. The study of cationic and anionic contributions has been done through the use of salts with varying cationic and anionic nature. In order to study anion specific effects on DNA binding by Klenow and Klentaq, the following potassium salts were used for salt linkage analysis: chloride, L-glutamate, D-glutamate, acetate, aspartate, and fluoride.
Proton linkage analysis was performed by obtaining $K_d$ at various pH points within the range 6 to 8.5. The number of thermodynamically linked protons exchanged, $\Delta n_{H^+}$, is given by the slope of the plot of $\log(1/K_d)$ vs. pH:

$$\frac{d \log(1/K_d)}{d \text{pH}} = \Delta n_{H^+} \quad \text{(Eq. 8)}$$

Proton linkage plots were obtained in 500mM KCl or Kglutamate for Klenow, and 100mM KCl or Kglutamate for Klentaq. Glutamic acid and HCl were used to pH respective buffers.

Osmotic pressure linkage was analyzed by obtaining association constants as a function of osmotic pressure ($\Pi$) induced by varying amounts of an osmolyte. Such analysis has been widely used to estimate changes in hydration associated with macromolecular processes\textsuperscript{125; 126}. It is based on the assumption that the presence of an osmolyte which is excluded (sterically or chemically) from macromolecular surfaces will result in a region of the water of hydration with higher water activity than the bulk solution. The release of water from this osmotically stressed region during macromolecular processes is entropically favorable. The favorable entropic contribution increases as the bulk water activity is decreased with increasing osmolyte concentration\textsuperscript{125; 126}. This technique has been applied to protein-DNA interactions, in particular for the comparative study of hydration changes associated with sequence-specific and non sequence-specific DNA binding\textsuperscript{127; 128; 129; 130; 131; 132}.

It is necessary to test for the size and nature of the osmolyte in order to maximize the confidence in the obtained value. The size of the osmolyte is a primary concern in the determination of the hydration change associated with a macromolecular process. Indeed, at a constant osmotic pressure, a polymeric osmolyte of increasing size can be further excluded from the binding interface by steric exclusion and therefore lead to enhanced protein-DNA binding if the latter is associated with water release. Furthermore, the increasing size of the osmolyte can
enhance DNA binding through macromolecular crowding. Therefore, at a constant osmotic pressure, polyethylene glycols of various sizes (MW: 200 Da to 10000 Da) were used to determine the osmolyte size at which the equilibrium constant was independent of the size of the osmolyte. Polyethylene glycols have been widely used for the estimation of hydration changes associated with macromolecular processes due to the steric nature of their exclusion from protein surfaces. Nevertheless, the chemical nature of the osmolyte must also be varied in order to help rule out the possibility of specific interactions between the osmolyte and the macromolecular surfaces resulting in the shift in protein-DNA equilibrium. In this study, ficoll 70 kDa, a polymer of sucrose was used to confirm the thermodynamically linked water release obtained by polyethylene glycol.

The hydration change associated with protein-DNA binding is estimated by the slope of:

\[
\frac{d}{d \Pi} \left( \frac{k_B T \ln(K_{\Pi} / K_0)}{\Pi} \right) = \Delta V_{H_2O} = \Delta n_{H_2O} \times 30 \, \text{Å}^3
\]  
(Eq. 9)

where \( k_B \) is Boltzmann’s constant, \( T \) is the temperature, \( K_{\Pi} \) is the association constant at osmotic pressure \( \Pi \) resulting from the osmolyte, \( K_0 \) is the association constant in the absence of osmolyte and \( \Delta V_{H_2O} \) is the volume of thermodynamically linked water released (or taken up) upon DNA binding. The number of waters released or taken up, \( \Delta n_{H_2O} \), is calculated from DVH2O using a molecular volume of 30Å³ per water molecule. Osmotic pressure was measured using a Wescor VAPRO vapor pressure osmometer (Logan, Utah). The hydration change associated with Klenow-DNA binding was obtained in 500mM KCl and 800mM Kglutamate while that of Klentaq was determined in 125mM KCl and 250mM Kglutamate.

2.3.1.3 Non-Linear Thermodynamic Salt Linkage Analysis
To account for non-linearity of salt linkage analysis, a non linear analysis was performed which incorporates a possible osmotic contribution from salt. The thermodynamic salt linkages incorporating the contribution of ion release and osmotically induced hydration change was derived using previously described approaches\textsuperscript{111,112}. The analysis is derived from the following reaction equation describing the exchange of $\Delta n_{A^-}$ anions $A^-$, $\Delta n_{C^+}$ cations $C^+$ and $\Delta n_w$ waters upon dissociation of the protein-DNA complex PD.

$$ PD + \Delta n_{A^-} A^- + \Delta n_{C^+} C^+ + \Delta n_w H_2O $$

$$ \leftrightarrow PA_{\Delta n_{A^-}} (H_2O)_{\Delta n_{A^-}} + DC_{\Delta n_{C^+}} (H_2O)_{\Delta n_{C^+}} $$

(Eq. 10)

The hydration change ($\Delta n_w$) represents the exchange of solute/salt-inaccessible hydration water associated with the free protein $\Delta n_{w_p}$ or the free DNA $\Delta n_{w_d}$, with $\Delta n_w = \Delta n_{w_p} + \Delta n_{w_d}$. The thermodynamic participation of $\Delta n_w$ results from differences in water activity of the salt/solute-inaccesible water of hydration of the macromolecules and the bulk water activity. The bulk water activity is decreased upon increasing the salt concentration resulting in the osmotic stress of the solute/salt-inaccessible hydration water. Therefore, if a salt results in an osmotically stressed water of hydration, the release of that water of hydration ($\Delta n_w$) upon DNA binding is favorable as salt concentration increases.

The equilibrium association constant for the reaction is expressed as:

$$ K_a = 1 / K_d = \frac{[PD][A^-]^{\Delta n_{A^-}} [C^+]^{\Delta n_{C^+}} [H_2O]^{\Delta n_w}}{[PA_{\Delta n_{A^-}}][DC_{\Delta n_{C^+}}]} = \frac{[PD][Salt]^{\Delta n_{Salt}} [H_2O]^{\Delta n_w}}{[PA_{\Delta n_{A^-}}][DC_{\Delta n_{C^+}}]} $$

(Eq. 11)

In order to describe the equilibrium binding constant as a function of salt concentration, the water concentration ([H$_2$O]) was expressed as a function of salt concentration using the
activity of water, $a_w$. The activity of water is directly to the osmolarity of the solution as shown in equation 12:

$$osm = -55.5 \ln(a_w) \iff a_w = e^{\left(\frac{osm}{55.5}\right)} \quad (Eq. \, 12)$$

The osmolarity of a solution is experimentally measurable using a vapor pressure osmometer (Wescor VAPRO vapor pressure osmometer). The osmolarity of solutions buffered with 10mM Tris, pH 7.9 with increasing concentrations of KCl or Kglutamate salts up to 1.5M was measured. The resulting relationship between the osmolarity and salt concentration was linear, and the linear regression yielded the slope $\alpha$ and the intercept $\beta$ (Eq. 13):

$$osm = \alpha[CA] + \beta \quad (Eq. \, 13)$$

These parameters were fixed in the expression of water activity as a function of salt concentration (Eq. 14) and in the non-linear linkage model (Eq. 16).

$$a_w = e^{\left(\frac{\alpha[CA]+\beta}{55.5}\right)} \quad (Eq. \, 14)$$

The logarithmic expression of the equilibrium binding constant expressed as a function of salt concentration (Eq. 11) yields the non-linear linkage model as follows:

$$\ln \left(\frac{[PD][Salt]}{[PA_{\Delta_n_{w}}][DC_{\Delta_n_{c}}]}\right) = \ln \left(\frac{[PD]}{[PA_{\Delta_n_{w}}][DC_{\Delta_n_{c}}]}\right) + \ln [Salt]^{\Delta n_{w}} + \ln(a_w^{\Delta n_{c}}) \quad (Eq. \, 15)$$

The relationship described in equation 14 is incorporated and the salt-independent reference equilibrium binding constant was expressed as $K_{d}^{ref}$ to obtain the final expression used to fit the non-linear thermodynamic salt linkages:
\[ \ln(1/K_d) = \ln\left(\frac{1}{K_d^{\text{ref}}}\right) + \Delta n_{\text{ions}} \ln[\text{salt}] + \Delta n_w (\frac{a_e \ln[\text{salt}]}{55.5} + \beta) \]  
(Eq. 16)

The fitting was performed by floating the \( \ln(1/K_d^{\text{ref}}) \), \( \Delta n_{\text{ions}} \) and \( \Delta n_w \) while \( \alpha \) and \( \beta \) are fixed to experimentally determined values. The parameters yielded were largely unaffected by variations in the initial guesses for the parameters floated. Indeed, the fit converged to the reported values for any initial guess other than 0. When an initial guess of 0 is entered for any of the floated parameters, the fit does not converge.

### 2.3.1.4 Data Analysis for Extended Salt Addition Titrations

Salt addition titrations normally, in chloride salts, produce an effectively inverted binding curve as salt displaces the protein from the DNA. In this dissertation, however, a secondary re-association of the protein and DNA occurs in high concentrations of glutamate. In order to fit and analyze these extended salt addition titrations, we expressed the fractional saturation of DNA with protein as a function of salt concentration.

The equilibrium association constant (Eq. 11) for the reaction can be rearranged as follows:

\[ [DC_{\Delta n_P}] = \frac{[PD][Salt]^\Delta n_{\text{water}}[H_2O]^\Delta n_w}{[PA_{\Delta n_{H}}]K_A} \]  
(Eq. 17)

The fractional DNA saturation is expressed as:

\[ \theta = \frac{[PD]}{[PD] + [D]} = \frac{[PD]}{[PD] + \frac{[PD][Salt]^\Delta n_{\text{water}}[H_2O]^\Delta n_w}{[PA_{\Delta n_{H}}]K_A}} \]  
(Eq. 18)
Here again, the thermodynamic activity of water was expressed as a function of salt concentration and consequently the fractional saturation was fit to the following equation:

\[
\theta = \frac{[PA_{\Delta n}]}{[PA_{\Delta n}][K_A]} \left(\frac{\alpha [Salt] + \beta}{55.5}\right)^{\Delta n_{sw}}
\]  

(Eq. 19)

2.3.2 Analysis of Circular Dichroism Data

Apparent melting temperatures (Tm) were determined in the absence of salt and in the presence of 100mM KCl, KF and Kglutamate. The obtained thermal unfolding curves were normalized and fitted to a modified version of the van’t Hoff equation which simultaneously fits for the native baseline, the transition and the denatured baseline:

\[
\Delta \varepsilon = (m_nT + b_n) + (m_dT + b_d)(\frac{e^{\frac{-\Delta H(1-T_m)}{RT}}}{1 + e^{\frac{-\Delta H(1-T_m)}{RT}}})
\]  

(Eq. 20)

where \(m_n\) and \(m_d\) are the slopes of the native and denatured baselines respectively, \(b_n\) and \(b_d\) are the Y intercept of the native and denatured state baselines, R is the gas constant, T is the temperature, \(\Delta H\) is the apparent change in enthalpy and \(T_m\) is the apparent melting temperature.

2.3.3 Analysis of Nucleotide Incorporation Data

The analysis procedure used for the nucleotide incorporation assay was developed by Hiromi Brown (unpublished work). The fraction of radioactive dATP incorporated was calculated by adjusting the raw scintillation count for background and total radioactivity using the following relationship:

\[
X = \frac{\text{Sample count (cpm)} - \text{Background count (cpm)}}{\text{Total count (cpm)}}
\]  

(Eq. 21)
The molar amount of total nucleotides incorporated was then calculated as follows:

\[
\text{pmoles of dNTPs incorporated} = X \times 150 \mu M \text{ dATP} \times 25 \mu L \text{ (reaction volume)} \times 4
\]

(Eq. 22)

This equation assumes that the incorporation of dATP amounts to, in average, 25% of all nucleotide incorporated.

In order to obtain the steady state rate of nucleotide incorporation, the molar amount of total dNTPs incorporated at each time point was plotted as a function of time. The steady state rate was determined from the slope of this relationship:

\[
\text{Steady State rate} = \frac{d (\text{pmoles dNTPs incorporated})}{d t}
\]

(Eq. 23)

The steady state rate was used as an indicators of the enzymatic activity and obtained in various concentrations of KCl and Kglutamate. For the purpose of correlating salt dependent behavior of DNA binding and enzymatic activities, these parameters (binding and activity) were individually normalized and plotted as a function of salt concentration.
CHAPTER 3: RESULTS

3.1 Linear Thermodynamic Linkage Analysis

3.1.1 Anion Dependence of Salt Linkage Is Observed

Protein DNA interactions are highly regulated by the salt concentration and type present in solution. Of particular physiological relevance is the effect of the substitution of chloride by glutamate and its consequence on the salt dependence of DNA binding. In our studies, salt effects on DNA binding by Klenow and Klentaq polymerases were studied by obtaining equilibrium dissociation constant as a function of salt concentration. A series of representative forward equilibrium titrations of Klenow in potassium glutamate is shown in Figure 5. The DNA binding affinities of Klenow and Klentaq were derived from the fit to the single site binding isotherm and plotted in salt linkage plots to obtain the linked ion release, \( \Delta n_{\text{ions}} \). 

Figure 5: Representative forward equilibrium titrations of Klenow in increasing concentrations of KGlutamate. All titrations contained 1nM ROX-labeled 13/20mer. The fits to equation 2 yielded the equilibrium dissociation constants (\( K_d \)) which are tabulated for both proteins in various salts in Table 1 and plotted in linkage plots in Figure 6.
variety of anionic salts were used in an attempt to estimate the relative contributions from anions and cations to \( \Delta n_{\text{ions}} \). All the parameters obtained (\( K_d \), \( \Delta n_{\text{ions}} \) and \( \Delta G^\circ_{1M} \)) are tabulated in Table 1 and the salt linkage plot is displayed in Figure 6. Figure 6 shows a standard thermodynamic salt linkage plot over a relatively restricted and linear response range. As will be shown later, extension of the examined salt range reveals significant non-linearity in the presence of glutamate but not chloride salts. In this section, however, the standard linear analysis and its implications will be discussed first.

Several observations can be made from the obtained data. First, similar submicromolar DNA binding affinities by Klenow and Klentaq are observed in different salt concentration ranges, irrespective of the nature of the anion. In KCl, Klentaq binds primed-template DNA with nanomolar affinity in 50-175mM salt (-3<ln[Salt]<-1.7). On the other hand, Klenow binds with nanomolar affinity from 200 up to 500mM KCl (-1.6<ln[Salt]<-0.7) (Table 1). Consequently, at any particular salt concentration Klenow binds \( \sim 150 \) fold stronger than Klentaq to the same DNA. These differences highlight the fact that DNA binding by Klenow persists into much higher salt concentration than by Klentaq (Figure 6).

Second, at all salt concentrations, the DNA binding affinities of both Klenow and Klentaq are also dependent on the nature of the anion present in solution. At any particular salt concentration, the hierarchy of DNA binding affinity enhancement follows the order Cl<acetate< glutamate (D or L isomer) ~ fluoride ~ aspartate invariably for each protein (Figure 6). This trend correlates well with previous studies involving DNA binding systems 93; 100; 101; 102; 135.

Third, the thermodynamically linked ion release of DNA binding is more significantly anion specific for Klenow than for Klentaq. The type of anion present in solution more significantly alters the \( \Delta n_{\text{ions}} \) of Klenow: the presence of glutamate (either isomer), aspartate or fluoride reduces \( \Delta n_{\text{ions}} \) by more than half compared to chloride and acetate. In chloride and
Table 1: The equilibrium dissociation constants ($K_d$) of Klenow and Klentaq to ROX-labeled 13/20mer. The $K_d$ was determined in various salt concentrations and types. The salt linkage analysis yielded the thermodynamically linked ion release ($\Delta n_{ions}$) and the Gibbs free energy of binding at 1M ($\Delta G^o_{1M}$).

<table>
<thead>
<tr>
<th>Salt type</th>
<th>[Salt] $M$</th>
<th>$K_d$ nM</th>
<th>$\Delta n_{ions}$</th>
<th>$\Delta G^o_{1M}$ kcal/mol</th>
<th>[Salt] $M$</th>
<th>$K_d$ nM</th>
<th>$\Delta n_{ions}$</th>
<th>$\Delta G^o_{1M}$ kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>0.25</td>
<td>5.8 ± 0.2</td>
<td>-4.9 ± 0.2</td>
<td>-7.3</td>
<td>0.05</td>
<td>5.7 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>12.8 ± 0.5</td>
<td></td>
<td></td>
<td>0.075</td>
<td>18.1 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td>27.3 ± 1.4</td>
<td>-4.9 ± 0.2</td>
<td>-7.3</td>
<td>0.1</td>
<td>47.2 ± 2</td>
<td>-3.4 ± 0.1</td>
<td>-5.3</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>53.3 ± 2.5</td>
<td></td>
<td></td>
<td>0.125</td>
<td>96.4 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>86.0 ± 5</td>
<td></td>
<td></td>
<td>0.15</td>
<td>215.0 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>183.6 ± 12</td>
<td></td>
<td></td>
<td>0.175</td>
<td>401.0 ± 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K acetate</td>
<td>0.3</td>
<td>5.1 ± 0.2</td>
<td>-4.4 ± 0.4</td>
<td>-8.1</td>
<td>0.1</td>
<td>12.1 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td>13.4 ± 0.7</td>
<td></td>
<td></td>
<td>0.125</td>
<td>34.7 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>17.1 ± 1.3</td>
<td>-4.4 ± 0.4</td>
<td>-8.1</td>
<td>0.15</td>
<td>66.3 ± 2.6</td>
<td>-3.8 ± 0.2</td>
<td>-5.6</td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>35.9 ± 1.1</td>
<td></td>
<td></td>
<td>0.175</td>
<td>98.0 ± 4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>49.8 ± 2.1</td>
<td></td>
<td></td>
<td>0.2</td>
<td>180.1 ± 7.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K aspartate</td>
<td>0.4</td>
<td>4.6 ± 0.2</td>
<td>-2.1 ± 0.4</td>
<td>-10.2</td>
<td>0.1</td>
<td>8.2 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>9.6 ± 0.4</td>
<td></td>
<td></td>
<td>0.135</td>
<td>27.6 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>10.4 ± 0.7</td>
<td>-2.1 ± 0.4</td>
<td>-10.2</td>
<td>0.175</td>
<td>50.8 ± 1.9</td>
<td>-3.2 ± 0.1</td>
<td>-6.6</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>15.9 ± 0.8</td>
<td></td>
<td></td>
<td>0.25</td>
<td>170.3 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>19.8 ± 1.4</td>
<td></td>
<td></td>
<td>0.325</td>
<td>356.7 ± 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KF</td>
<td>0.4</td>
<td>6.4 ± 0.3</td>
<td>-2.7 ± 0.2</td>
<td>-9.7</td>
<td>0.1</td>
<td>10.0 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>13.7 ± 0.4</td>
<td></td>
<td></td>
<td>0.135</td>
<td>28.3 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>22.0 ± 1.3</td>
<td>-2.7 ± 0.2</td>
<td>-9.7</td>
<td>0.175</td>
<td>53.9 ± 1.5</td>
<td>-3.3 ± 0.1</td>
<td>-6.4</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>33.5 ± 1.6</td>
<td></td>
<td></td>
<td>0.25</td>
<td>194.6 ± 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>39.8 ± 3.4</td>
<td></td>
<td></td>
<td>0.325</td>
<td>500.1 ± 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K (L)-Glu</td>
<td>0.2</td>
<td>1.8 ± 0.08</td>
<td>-2.1 ± 0.1</td>
<td>-9.8</td>
<td>0.1</td>
<td>11.6 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>4.8 ± 0.2</td>
<td></td>
<td></td>
<td>0.135</td>
<td>26.9 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>11.6 ± 0.5</td>
<td></td>
<td></td>
<td>0.175</td>
<td>77.0 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>14.1 ± 1.1</td>
<td>-2.1 ± 0.1</td>
<td>-9.8</td>
<td>0.25</td>
<td>184.5 ± 9</td>
<td>-3.1 ± 0.1</td>
<td>-6.6</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>22.4 ± 1.9</td>
<td></td>
<td></td>
<td>0.325</td>
<td>447.5 ± 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>28.7 ± 1.7</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>32.0 ± 2.5</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K(D)-Glu</td>
<td>0.4</td>
<td>13.1 ± 0.4</td>
<td>-1.7 ± 0.1</td>
<td>-9.8</td>
<td>0.1</td>
<td>13.0 ± 0.1</td>
<td>-2.8 ± 0.1</td>
<td>-7.0</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>26.5 ± 1.8</td>
<td></td>
<td></td>
<td>0.325</td>
<td>346.6 ± 14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
acetate, 4.5~5 ions are released upon binding as opposed to 2~2.5 ions released in the presence of glutamate, aspartate or fluoride (Table 1). Consequently, Klenow-DNA binding affinity is 3 fold stronger in glutamate than chloride at 300mM salts and 12 fold stronger at 500mM. For Klenow, low nanomolar equilibrium dissociations constants (~30nM) are detected in up to 800mM glutamate. This effect is considerably less pronounced in Klentaq. Klentaq’s \( \Delta n_{\text{ions}} \) in all studied anionic salts was determined to be 3~4 ions (Table 1). As a result, Klenow is more salt sensitive than Klentaq in KCl (4.9 versus 3.4 linked ion release) yet the opposite is true when chloride is replaced by glutamate (2.1 versus 3.1 linked ion release) (Table 1). Similar decreases in linked ion release have been reported in DNA binding proteins.

![Figure 6: Salt linkage of Klenow and Klentaq DNA polymerase in the presence of various potassium salts. Shown here is the linear regression analysis of the salt linkage of Klenow (empty symbols) and Klentaq (filled symbols) in the various anionic salts listed. The dissociation constants (K\(_d\)) at each salt concentration and the parameters obtained by linear regression (\( \Delta n_{\text{ions}} \) and \( \Delta G^{\circ}_{1M} \)) are listed in Table 1.](image)
for glutamate (and fluoride) in various *E.coli* systems \(^99; 100; 101; 102\). The results reported for Klenow confirm what has been termed “the glutamate effect”. Interestingly, the glutamate effect on Δ\(n_{\text{ions}}\) is apparently not significant in Klentaq. This observation is mirrored in the Gibbs free energy of binding at 1M (Δ\(G^o_{1M}\)). The Δ\(G^o_{1M}\) is obtained by extrapolating the salt linkage to 1M (ln[Salt]=0) where one obtains the apparent non-electrostatic contribution to DNA binding \(^{19}\). We have determined that both proteins show some dependence of the Δ\(G^o_{1M}\) on the type of anion present and the dependence displayed correlates well with the anion dependence of Δ\(n_{\text{ions}}\). For Klenow, it was found that the substitution of chloride by glutamate (aspartate, or fluoride) yields an enhancement in Δ\(G^o_{1M}\) of about ~2 to 2.5 kcal/mol. In contrast, the Δ\(G^o_{1M}\) of Klentaq is less sensitive of the type of anion present with similar substitutions leading to an enhancement of ~1 kcal/mol (Table 1).

Table 1 also shows that the Δ\(G^o_{1M}\) is consistently more favorable for Klenow than Klentaq. The difference in Δ\(G^o_{1M}\) between Klenow DNA binding and Klentaq DNA binding ranges from 1.7 kcal/mol in KCl to ~3 kcal/mol in Kglutamate, Kaspartate and KF (Table 1).

**3.1.2/ Proton Linkage Is Also Anion Dependent in Klenow**

DNA binding by Klenow and Klentaq polymerases was also examined as a function of pH. Table 2 lists the equilibrium dissociation constants (\(K_d\)) for Klenow and Klentaq’s interaction with the 13/20mer construct at different pHs and in the presence of KCl or Kglutamate. Proton linkage plots are shown in Figure 7, and the thermodynamically linked proton exchange associated with DNA binding is listed in Table 2. Klentaq DNA binding remained in the nanomolar range across the pH range from 6 to 8.5 in 100mM potassium salt. As the pH is increased from 6 to 8.5, a moderate increase in equilibrium dissociation constant is observed. Furthermore, the slope of the proton linkage reveals that +0.3 protons are taken up upon Klentaq-DNA binding and that the proton linkage observed is not dependent on the
Table 2: Equilibrium dissociation constants ($K_d$) of the interaction of Klenow and Klentaq with primed-template at varying pH. The dissociation constants were obtained in the presence of KCl or Kglutamate. The resulting proton linkage ($\Delta n_{H^+}$) is listed for both proteins in the two anionic salts.

<table>
<thead>
<tr>
<th>Salt type</th>
<th>pH</th>
<th>$K_d$</th>
<th>$\Delta n_{H^+}$</th>
<th>pH</th>
<th>$K_d$</th>
<th>$\Delta n_{H^+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>157.2 ± 11.6</td>
<td></td>
<td>6</td>
<td>7.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>6.5</td>
<td>149.1 ± 8.8</td>
<td></td>
<td>6.5</td>
<td>17.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>141.5 ± 21.2</td>
<td>0.06 ± 0.04</td>
<td>7</td>
<td>31.1 ± 0.9</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>206.2 ± 28.9</td>
<td></td>
<td>7.5</td>
<td>42.6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>183.6 ± 42.0</td>
<td></td>
<td>7.9</td>
<td>47.2 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>KGlu</td>
<td>6</td>
<td>4.8 ± 0.2</td>
<td></td>
<td>6</td>
<td>2.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>4.2 ± 0.1</td>
<td></td>
<td>6.5</td>
<td>3.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>6.5 ± 0.4</td>
<td>0.30 ± 0.04</td>
<td>7</td>
<td>6.3 ± 0.4</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>9.8 ± 0.7</td>
<td></td>
<td>7.5</td>
<td>8.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>14.1 ± 1.2</td>
<td></td>
<td>7.9</td>
<td>11.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>22.7 ± 1.8</td>
<td></td>
<td>8.5</td>
<td>16.5 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

All $K_d$ values listed in this table have been adjusted with respect to the standard $K_d$ obtained at pH 7.9 for the salt linkage (Table 1). Errors reported are associated with the parameter prior to adjustment.

nature of the anion present in solution (Figure 7B, Table 2). Nanomolar range DNA binding affinity was also observed for Klenow within the pH 6 to 8.5 range. In 500mM KCl, the observed dissociation constant does not significantly increase with increasing pH. Consequently, the proton linkage was determined to be very low at ~0.06 protons taken up during binding (Table 2). However, the pH dependence of binding seems to be affected to a greater extent by the presence of glutamate. Indeed, we observe a more pronounced increase in $K_d$ in the presence of Kglutamate as the pH increases. Consequently, the proton linkage increases five fold
Figure 7: Proton linkage of DNA binding by Klenow (panel A) and Klentaq (panel B). The equilibrium dissociation constants were obtained in the presence of 500mM salt (KCl or Kglutamate) for Klenow and 100mM salt (KCl and Kglutamate) for Klentaq.

(~0.3 protons) when KCl is substituted by Kglutamate (Figure 7A, Table 2).

The amplitude of all proton linkages obtained (<1) suggests that the pH dependence of DNA binding is due to the gradual deprotonation of basic residues on the protein surface involved in DNA binding as pH is increased. Moreover, Klenow and Klentaq display a different
anion dependence on the proton linkage. The substitution of chloride by glutamate significantly alters the proton linkage in Klenow but has no effect on the proton linkage in Klentaq.

3.1.3/ Hydration Change Measured by the Osmotic Stress Technique

To complete the characterization of thermodynamic linkages of DNA binding properties as a function of solution conditions, the osmotic stress technique was used to determine the hydration change associated with DNA binding by Klenow and Klentaq. The extent of the change in hydration associated with DNA binding was obtained and the effect of glutamate on this parameter was also examined. In order to increase the confidence in the obtained parameters, several controls were performed with regard to chemical nature and size of the stressing solute.

A primary concern is the molecular size of the stressing solute. Polyethylene glycol, a polymeric osmolyte routinely used in osmotic stress experiments\textsuperscript{125; 126}, was used to probe the effect of the solute size. The stressing solute should be bulky enough to be sterically excluded from the binding cleft as well as all other protein surface crevasses in

\[ \text{Table 3: Equilibrium dissociation constants of Klenow and Klentaq’s interaction to primed template obtained in the presence of PEG. At constant osmolarity, PEG of increasing molecular weights were used to obtain the K}_d. \text{ DNA binding titrations were performed in 500mM KCl for Klenow and 100mM KCl for Klentaq.} \]

\[
\begin{array}{|c|c|c|c|c|c|c|}
\hline
\text{PEG molecular weight (Da)} & \text{[Solute]} \, \%w/v & \text{K}_d \, nM & \\
\hline
\text{No PEG} & 0 & 183.6 \pm 12 & \\
\text{200} & 3.2 & 80.4 \pm 11 & \\
\text{1000} & 7 & 40.0 \pm 6.4 & 1000 \, 6 & 38.7 \pm 0.4 & \\
\text{3000} & 9 & 21.4 \pm 3.8 & 3000 \, 9 & 22.4 \pm 0.8 & \\
\text{6000} & 10 & 18.9 \pm 2.3 & 6000 \, 10 & 18.3 \pm 0.4 & \\
\text{8000} & 10 & 17.8 \pm 3.4 & 8000 \, 10 & 17.9 \pm 0.4 & \\
\text{10000} & 10 & 17.6 \pm 3.7 & - & - & - \\
\hline
\end{array}
\]
order to maximize the amount of osmotically stressed water displaced upon DNA binding. However, increased size of the solute can cause macromolecular crowding which results in increased apparent affinity due to increased effective concentrations of the reactants. In order to determine the effect of solute size on binding affinity, at constant osmotic pressure (1120 mOsm for Klenow and 320 mOsm for Klentaq), the equilibrium dissociation constants of Klenow and Klentaq-DNA binding in the presence of PEG of increasing size were obtained (Figure 8A, Table 3). The results obtained show that above 6000 molecular weight, further increasing the size of PEG has little additional effect on the equilibrium of binding of both Klenow and Klentaq to DNA. This observation suggested that PEGs above a molecular weight value of 6000 Da were excluded to a similar extent from the binding cleft. Furthermore, the plateau observed for PEGs beyond 6000 Da also suggests that macromolecular crowding effects do not significantly alter the equilibrium of binding in the range of osmolyte size that was probed. Therefore, we used PEG 6000 Da to determine the hydration change upon DNA binding.

However, we also determined the extent of hydration change upon DNA binding in Ficoll 70kDa, another bulky polymeric (polymer of sucrose) osmolyte. The hydration changes associated with Klenow and Klentaq-DNA binding in the presence of PEG 6000 Da or Ficoll 70kDa were first measured in KCl (Figure 8B, Table 4 and 5). In 500 mM KCl, we report the release of ~479 waters in PEG 6000 (the value reported here is an average of three determinations) and ~537 waters in Ficoll 70 (the value reported here is an average of two determinations) upon Klenow-DNA binding. In 125 mM KCl, Klentaq-DNA binding yields ~521 waters released in PEG 6000 Da and ~642 waters in Ficoll 70kDa. For the respective proteins, the hydration change values obtained in the chemically different solutes are similar and within statistical error, yielding a net water release of 508.5 ($\pm$ 29.5) waters for Klenow and ~581 ($\pm$ 53.8) waters for Klentaq.
Figure 8: Panel A: The dependence of DNA binding equilibrium association constant ($K_A$) of Klenow and Klentaq as a function of the PEG size. Increasing sizes of PEG were used at constant osmotic pressure. Panel B: Hydration change determined by the osmotic stress technique using two chemically different osmolytes (PEG 6000 Da and Ficoll 70 kDa) in the presence of KCl (125mM for Klentaq or 500mM for Klenow) or Kglutamate (250mM for Klentaq and 800mM for Klenow).
Table 4: Hydration change associated with the DNA binding by Klenow to the 13/20mer construct. Polyethylene glycols of varying size and Ficoll 70kDa were used. The experiments were performed in 500mM and 800mM Kglutamate. The apparent numbers of water released are listed.

<table>
<thead>
<tr>
<th>Solute type</th>
<th>KCl (500mM)</th>
<th></th>
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<th>KGl (800mM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>[Solute] %w/v</td>
<td>Π Osm</td>
<td>Kd nM</td>
<td>ΔnH2O</td>
<td>[Solute] %w/v</td>
<td>Π Osm</td>
<td>Kd nM</td>
<td>ΔnH2O</td>
</tr>
<tr>
<td>PEG 200 Da</td>
<td>0 0.93 370 ± 30</td>
<td>370 ± 30</td>
<td>109.1 ± 10.7</td>
<td>52.5 ± 3.7</td>
<td>0 1.59 29.4 ± 1.3</td>
<td>29.4 ± 1.3</td>
<td>114.5 ± 18.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 1.31 107.4 ± 11</td>
<td>107.4 ± 11</td>
<td>2.5 1.85 21.9 ± 1.6</td>
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<tr>
<td></td>
<td>10 1.71 46.4 ± 8.3</td>
<td>46.4 ± 8.3</td>
<td>5 2.17 16.2 ± 0.9</td>
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<tr>
<td></td>
<td>15 2.18 23.1 ± 2.3</td>
<td>23.1 ± 2.3</td>
<td>7.5 2.47 13.1 ± 0.7</td>
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<tr>
<td></td>
<td>20 2.73 9.8 ± 0.8</td>
<td>9.8 ± 0.8</td>
<td>10 2.82 11.4 ± 1.0</td>
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</tr>
<tr>
<td>PEG 1000 Da</td>
<td>0 0.93 370 ± 30</td>
<td>370 ± 30</td>
<td>293.2 ± 32.6</td>
<td>151.1 ± 14.1</td>
<td>0 1.59 29.4 ± 1.3</td>
<td>29.4 ± 1.3</td>
<td>151.1 ± 14.1</td>
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</tr>
<tr>
<td></td>
<td>5 1.07 80.2 ± 6.7</td>
<td>80.2 ± 6.7</td>
<td>2.5 1.72 18.5 ± 1.8</td>
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<td></td>
<td>10 1.23 41.5 ± 3.7</td>
<td>41.5 ± 3.7</td>
<td>5 1.91 13.7 ± 0.8</td>
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<tr>
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<td>15 1.45 19.0 ± 2.6</td>
<td>19.0 ± 2.6</td>
<td>7.5 2.09 7.4 ± 0.9</td>
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<tr>
<td></td>
<td>20 1.68 5.1 ± 0.3</td>
<td>5.1 ± 0.3</td>
<td>10 2.30 7.2 ± 0.7</td>
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<tr>
<td>PEG 6000 Da</td>
<td>0 0.93 370 ± 30</td>
<td>370 ± 30</td>
<td>480.6 ± 28.1</td>
<td>151.1 ± 14.1</td>
<td>0 1.59 32.3 ± 1.5</td>
<td>32.3 ± 1.5</td>
<td>151.1 ± 14.1</td>
<td></td>
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<tr>
<td></td>
<td>5 1.03 105.7 ± 10</td>
<td>105.7 ± 10</td>
<td>2.5 1.72 16.7 ± 1.1</td>
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<td>34.2 ± 4.8</td>
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<td>11.2 ± 0.8</td>
<td>7.5 2.04 8.5 ± 0.5</td>
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<tr>
<td>Ficoll 70 kDa</td>
<td>0 0.91 465.6 ± 30</td>
<td>465.6 ± 30</td>
<td>523.4 ± 59.3</td>
<td>136.1 ± 7.9</td>
<td>0 1.59 29.1 ± 1.6</td>
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<td>136.1 ± 7.9</td>
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<tr>
<td></td>
<td>5 0.94 421.5 ± 25</td>
<td>421.5 ± 25</td>
<td>2.5 1.66 23.7 ± 0.8</td>
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<td>179.3 ± 10</td>
<td>5 1.74 19.3 ± 1.0</td>
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<tr>
<td></td>
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<td>118.8 ± 5</td>
<td>7.5 1.79 17.9 ± 1.3</td>
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<td>52.4 ± 3.8</td>
<td>10 1.87 14.5 ± 2.7</td>
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<tr>
<td></td>
<td>24 1.25 23.4 ± 1.5</td>
<td>23.4 ± 1.5</td>
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</table>

* Data listed represents only one of multiple series performed (3 for PEG 6000Da and 2 for Ficoll 70kDa in the presence of KCl). Repeat series are tabulated in the appendix A. The values reported in this table are plotted in Figure 9 panel A and B. The average values of ΔnH2O are tabulated within Figure 9 and plotted in panel C and D. [49]
Table 5: Hydration change associated with the DNA binding by Klenow to the 13/20mer construct. Polyethylene glycols of varying size and Ficoll 70kDa were used. The experiments were performed in 500mM and 800mM Kglutamate. The apparent numbers of water released are listed.

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<th>Solute type</th>
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<th>Kd</th>
<th>ΔnH2O</th>
<th>[Solute]</th>
<th>Π</th>
<th>Kd</th>
<th>ΔnH2O</th>
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<td>PEG 200 Da</td>
<td>0 0.25</td>
<td>117.6 ± 7</td>
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<td>0 0.47</td>
<td>127.5 ± 5</td>
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<td></td>
<td>5 0.56</td>
<td>65.4 ± 5.1</td>
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<td></td>
<td>5 0.86</td>
<td>87.4 ± 6.4</td>
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<td>10 0.97</td>
<td>30.5 ± 1.5</td>
<td>110.8 ± 1.7</td>
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<td>55.7 ± 4.2</td>
<td>71.8 ± 4.1</td>
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<td>15 1.36</td>
<td>13.5 ± 0.4</td>
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<td>15 1.80</td>
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<td>5.1 ± 0.3</td>
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<td></td>
<td>20 2.37</td>
<td>11.6 ± 1.2</td>
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<tr>
<td>PEG 1000 Da</td>
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<td>117.6 ± 7</td>
<td></td>
<td></td>
<td>0 0.47</td>
<td>127.5 ± 5</td>
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<td>5 0.34</td>
<td>60.0 ± 3.3</td>
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<td></td>
<td>5 0.64</td>
<td>99.2 ± 5.7</td>
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<td></td>
<td>10 0.47</td>
<td>19.8 ± 0.5</td>
<td>295.0 ± 39.0</td>
<td></td>
<td>10 0.85</td>
<td>42.2 ± 3.5</td>
<td>152.9 ± 9.1</td>
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<td></td>
<td>15 0.62</td>
<td>10.1 ± 0.2</td>
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<td></td>
<td>15 1.10</td>
<td>25.2 ± 2.1</td>
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<td></td>
<td>20 0.81</td>
<td>5.7 ± 0.4</td>
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<td>20 1.41</td>
<td>10.5 ± 0.8</td>
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<tr>
<td>PEG 6000 Da</td>
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<td>117.6 ± 7</td>
<td></td>
<td></td>
<td>0 0.47</td>
<td>127.5 ± 5</td>
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<tr>
<td></td>
<td>5 0.28</td>
<td>66.5 ± 2.0</td>
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<td>5 0.57</td>
<td>93.1 ± 7.9</td>
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<tr>
<td></td>
<td>10 0.34</td>
<td>29.1 ± 0.6</td>
<td>521.1 ± 48.1</td>
<td></td>
<td>10 0.72</td>
<td>55.9 ± 3.5</td>
<td>173.6 ± 10.0</td>
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<td>15 0.45</td>
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<td>4.4 ± 0.1</td>
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<td></td>
<td>20 1.14</td>
<td>17.1 ± 1.0</td>
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</tr>
<tr>
<td>Ficoll 70 kDa</td>
<td>0 0.25</td>
<td>116.2 ± 7</td>
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<td></td>
<td>5 0.26</td>
<td>100.7 ± 5</td>
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<td></td>
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<tr>
<td></td>
<td>10 0.27</td>
<td>83.7 ± 5</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>15 0.29</td>
<td>59.5 ± 4.3</td>
<td>642.3 ± 96.2</td>
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<tr>
<td></td>
<td>20 0.31</td>
<td>39.7 ± 2.5</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>25 0.36</td>
<td>23.1 ± 2.9</td>
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</tbody>
</table>
The similarity of the hydration change observed in the presence of chemically different osmolyte weakens the probability that there are specific interactions of the solutes with any of the macromolecular surfaces and strengthens the assumption of chemical inertness of the solutes towards the protein surface, allowing greater confidence in the obtained water released values.

The hydration change associated with DNA binding was then estimated in the presence of glutamate to investigate a possible ‘glutamate effect’ on $\Delta n_{H2O}$. In Klenow, the hydration change upon DNA binding displays a large dependence on the type of anion present in solution. In 800mM K$_{glutamate}$, $\Delta n_{H2O}$ in PEG 6000 Da and Ficoll 70kDa were determined to be $\sim$151 and $\sim$136 waters respectively. The obtained $\Delta n_{H2O}$ values are within statistical error of each other and yield an average hydration change of $\sim$144 (± 8.1) waters associated with DNA binding (Figure 8B, Table 4). The substitution of chloride by glutamate thus results in a decrease in $\Delta n_{H2O}$ of $\sim$330 waters (or $\sim$70%), irrespective of the type of stressing solute used. This effect of glutamate on osmotic stress of Klenow-DNA binding is further evident upon changing the PEG size. Indeed, the presence of glutamate seems to attenuate the dependence of $\Delta n_{H2O}$ on the size of PEG. For Klenow DNA binding, the decrease in PEG size from PEG 6000 Da to PEG 200 Da, results in a 4.4 fold decrease of $\Delta n_{H2O}$ in KCl and 2.9 fold in K$_{glutamate}$, as shown in Figure 9, Table 4.

In Klenow, the dramatic ‘glutamate effect’ on $\Delta n_{H2O}$ complements the ‘glutamate effect’ observed on $\Delta n_{ions}$ and $\Delta n_{H+}$. Alternatively, whereas Klentaq does not display a glutamate effect on $\Delta n_{ions}$ or $\Delta n_{H+}$, the $\Delta n_{H2O}$ of Klentaq is anion dependent in a similar fashion to Klenow. In 250mM K$_{glutamate}$, DNA binding by Klentaq in PEG 6000 Da resulted in $\sim$174 waters released (Figure 8B, Table 5). The substitution of chloride by glutamate results in a decrease in $\Delta n_{H2O}$ of $\sim$350 waters (or $\sim$70%). The presence of glutamate also attenuates the PEG size dependence of $\Delta n_{H2O}$ in Klentaq as it does in Klenow, as shown in Figure 9 and table 5.
Figure 9: Hydration change associated to DNA binding by Klenow (panel A) and Klentaq (panel B) determined by the osmotic stress technique in the presence of KCl or Kglutamate. PEGs of varying size were used as stressing solute. All hydration change values are displayed in the tables. The hydration change values were plotted as a function of PEG size in panel C (Klenow) and panel D (Klentaq).
3.2/ Salt Effects on the Melting Temperature $T_m$

In order to examine the effect of salt concentration and type on the folding stability of Klenow and Klentaq, their apparent $T_m$ was determined in the absence of salt and in the presence of 100mM KF, 100mM Kglutamate (L and D isomers) and 100mM KCl using circular dichroism (Figure 10, Table 6). Glutamate, an optically active compound, absorbs strongly in the range of 210-220nm causing a very unstable CD signal. In order to obtain thermal unfolding curves in glutamate, CD signal of equal mixtures of L and D isomers were monitored at 227nm as opposed to 222nm for the other salts.

Table 6: Apparent melting temperatures ($T_m$) of Klenow and Klentaq obtained by thermal unfolding monitored by circular dichroism. The apparent $T_m$ was obtained in the absence of salt and 100mM KF, KCl and Kglutamate.

<table>
<thead>
<tr>
<th></th>
<th>Klenow</th>
<th>Klentaq</th>
</tr>
</thead>
<tbody>
<tr>
<td>No salt</td>
<td>$42.2 \pm 0.1 ^\circ$C</td>
<td>$98.6 \pm 0.1 ^\circ$C</td>
</tr>
<tr>
<td>KCl</td>
<td>$46.6 \pm 0.2 ^\circ$C</td>
<td>$98.9 \pm 0.1 ^\circ$C</td>
</tr>
<tr>
<td>KF</td>
<td>$46.7 \pm 0.2 ^\circ$C</td>
<td>$99.2 \pm 0.1 ^\circ$C</td>
</tr>
<tr>
<td>KGlut</td>
<td>$47.4 \pm 0.4 ^\circ$C</td>
<td>$99.6 \pm 0.1 ^\circ$C</td>
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</tbody>
</table>

The presence of salts during thermal unfolding enhanced aggregation of the unfolded state (data not shown) and therefore limited the salt concentration to 100mM. The aggregation of the unfolded state which was monitored by dynode voltage, was absent in the absence of salt but visible for both proteins in the presence of salts. While Klentaq aggregated immediately upon denaturing, the unfolding and aggregation transitions were clearly separate for Klenow.
Nevertheless, Klenow’s thermal unfolding is irreversible, indicating that all obtained $T_m$ values are apparent thermodynamic parameters as opposed to rigorous one. Apparent thermodynamic parameters are reproducible and precise, but may contain some energetic contributions from processes other than the primary process (such as aggregation).

Thermal unfolding curves and apparent $T_m$ values are reported in Figure 10 and Table 6. The apparent $T_m$ of Klenow in the absence of salt was determined to be 42.2°C. The addition of

![Thermal unfolding curves of Klenow (Panel A) and Klentaq (Panel B) monitored by CD. The thermal unfolding was monitored in the absence or presence of salt (100mM KCl, KF or Kglutamate)](image-url)
100mM salt stabilized Klenow by ~5°C invariably in all salts. However, there are no significant
differential effects observed upon substitution of the anion in solution; glutamate does not shift
Klenow’s apparent T_m significantly further than chloride (Figure 10A, Table 6). Klentaq’s
apparent T_m in the absence of salt was determined to be 98.7°C. In all the anionic salts used, the
addition of 100mM salt did not significantly alter the T_m of Klentaq (Figure 10B, Table 6).

3.3/ Extension of Salt Linkage Studies to High Salt Concentration

3.3.1/ Non-Linear Thermodynamic Linkage in the Presence of Kglutamate

The effect of glutamate on the hydration changes associated with DNA binding suggests that the
salt linkage data analysis must be extended to include a contribution from the exchange of water
from the binding interface. The salt concentration must also be experimentally extended to probe
the same thermodynamic space of bulk water activity as that of the osmotic stress experiment.
Therefore, the equilibrium dissociation constants for Klenow and Klentaq DNA binding in
Kglutamate were extended to 2.5 M. We observed that the K_d in both systems weakens up to
800mM Kglutamate with a maximal K_d of ~30nM for Klenow and ~1.9uM for Klentaq (Figure
11A and 11C, Table 7). Beyond 800mM, we observed that the K_d of the protein DNA binding
decreases down to 0.5 nM affinity in Klenow and to ~25nM for Klentaq (Figure 11B and 11D,
Table 7). The obtained equilibrium constants were incorporated in the salt linkage plots and
resulted in a significant deviation from linearity (Figure 12A). The extended salt linkage
displayed a biphasic behavior with a negative linkage at low salt and a positive linkage a high
salt. Along with halophilic PwTBP, Klenow and Klentaq constitute the only observed instances
of reversal of salt linkages at high salt. Klenow and Klentaq displayed similar curvature to one
another all throughout the salt concentration range tested. As a result, the large ΔΔG°(Kln-Ktq),
the binding affinity is seen to remain relatively constant at ~ -3kcal/mol throughout the entire salt
range (Figure 12B)
Figure 11: Individual fluorescence anisotropy titrations of Klenow DNA binding (panel A and B) and Klentaq DNA binding (panel C and D) at increasing Kglutamate concentration. Panel A and C show decreased binding affinity as [KGlut] is increased to 800mM and Panel B and D show increased binding affinity as [KGlut] is further increased up to 2M.

The linear fit of the thermodynamic salt linkages (Eq. 6) was modified to fit to the non-linear extended salt linkage by including an osmotic component which expresses the activity of water as a function of ln[KGlut] (Eq. 16).
Table 7: The equilibrium dissociation constants (Kd) of Klenow and Klentaq to ROX-labeled 13/20mer. The Kd was determined in various concentrations of Kglutamate. The non linear salt linkage analysis yielded the thermodynamically linked ion release ($\Delta n_{ions}$), the thermodynamically linked water release ($\Delta n_w$) and a reference Gibbs free energy of binding.

<table>
<thead>
<tr>
<th>[KGlut] M</th>
<th>$K_d$ nM</th>
<th>$\Delta n_{ions}$</th>
<th>$\Delta n_w$</th>
<th>$\Delta G^o_{ref}$ kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Klenow</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>1.8 ± 0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>4.8 ± 0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>11.6 ± 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>14.1 ± 1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>22.4 ± 1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.7</td>
<td>28.7 ± 1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>32.0 ± 2.5</td>
<td></td>
<td></td>
<td>-5.3 ± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>25.0 ± 4.3</td>
<td></td>
<td>-183.9 ± 10.0</td>
<td>-6.3</td>
</tr>
<tr>
<td>1.25</td>
<td>13.8 ± 1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.375</td>
<td>8.8 ± 0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>5.2 ± 0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.625</td>
<td>4.2 ± 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.48 ± 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Klentaq</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>11.6 ± 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.135</td>
<td>26.9 ± 0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.175</td>
<td>77.0 ± 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>184.5 ± 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.325</td>
<td>447.5 ± 15</td>
<td></td>
<td></td>
<td>-4.3 ± 0.2</td>
</tr>
<tr>
<td>0.5</td>
<td>921.0 ± 31</td>
<td></td>
<td>-139.7 ± 5.6</td>
<td>-4.8</td>
</tr>
<tr>
<td>0.8</td>
<td>1782.8 ± 140</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1695.4 ± 121</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td>1008.4 ± 38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.25</td>
<td>33.0 ± 2.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>26.7 ± 3.0</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
\[ \ln\left(\frac{1}{K_d}\right) = \ln\left(\frac{1}{K_{d_{ref}}}\right) + \Delta n_{\text{ions}} \ln [\text{salt}] + \Delta n_{\text{w}}\left(-\frac{\alpha e^{\ln [\text{salt}]} + \beta}{55.5}\right) \quad \text{(Eq. 16)} \]

The resulting fitted parameters are listed in Table 7. As described in chapter 2, equation 16 is derived from the expression of the equilibrium constant which takes into account the possible thermodynamic participation of both ions and water. While the ionic concentration are expressed in M units, the participation of water is accounted for by the direct measurement of the osmolarity of the solution using vapor pressure osmometer and converted into the thermodynamic activity of water. The water activity was expressed as a function of molar salt concentration by measuring the osmolarity of increasing salt concentration solutions and obtaining the slope (\(\alpha\)) and intercept (\(\beta\)) by linear regression (Figure 13). The obtained parameters (\(\alpha\) and \(\beta\)) for Kglutamate were fixed in the non linear fit to the extended salt linkage (Figure 12).

The model used describes the ionic contribution which is predominant at low salt concentration and is increasingly unfavorable to DNA binding as salt concentration is increased. This component yields the parameter \(\Delta n_{\text{ions}}\), the number of thermodynamically linked ion release. This parameter is derived from the slope of the transition at low salt, where the electrostatic effect is predominant and is therefore equivalent to \(\Delta n_{\text{ions}}\) in the linear analysis of salt linkage (Figure 6). The fit yields \(\sim 5.3\) ions displaced upon DNA binding by Klenow and \(\sim 4.3\) displaced for Klentaq-DNA binding (Table 7). This observation correlates well with the linear regression analysis that Klenow is more salt sensitive (higher salt linkage) than Klentaq in the presence of KCl (Table 1).

On the other hand, if the salt results in osmotically stressed water of hydration, the osmotic contribution grows increasingly favorable as salt concentration is increased. At higher
Figure 12: Extension of salt linkage for Klenow and Klentaq DNA binding after incorporation of titrations above 800mM Kglutamate. Panel A: The linkage displays non-linerarity. Non-linear fits parameters were obtained and displayed in Table 7. Panel B: The plots were converted to $\Delta G$ vs. $\ln[K\text{Glu}]$ and fit with the corresponding non-linear fit. Extrapolated $\Delta\Delta G$ values are plotted vs. $\ln[K\text{Glu}]$.
salt concentration, the osmotic component becomes predominant. It causes deviation from linearity and in strongly osmotic salts, such as glutamate, can eventually reverse the apparent salt linkage. From these parts of the dependence, we obtain $\Delta n_w$, the number of thermodynamically linked waters released. The statistical fit yields $\sim 196$ osmotically stressed water release associated with Klenow-DNA binding and $\sim 148$ osmotically stressed water release associated with Klentaq-DNA binding (Table 7). The results obtained are comparable to that obtained by the osmotic stress technique in Kglutamate and PEG 6000 Da (Table 4 and 5).

Figure 13: The osmotic pressure of the binding buffers (10mM Tris, pH 7.9) as a function of salt concentration (Kglutamate and KCl). The osmotic pressure was measured using a Wescor vapor pressure osmometer. The resulting slope and intercept ($\alpha$ and $\beta$) were used in the non linear fit to the extended salt linkage and salt addition.

The non linear analysis also yields a salt independent parameter indicative of a reference binding affinity analogous to the parameter used to obtain the $\Delta G^\circ_{1M}$ in the linear regression. In the non-linear fit, this thermodynamic parameter could be indicative of a salt/solvent independent intrinsic binding affinity of the protein and is $-6.3$ kcal/mol for Klenow and $-4.8$ kcal/mol.
kcal/mol for Klentaq (Table 7). This energetic difference could account for most of the observed
ΔΔG_{Kln-Ktq} which is constant ( -2.5 to -3 kcal/mol) throughout the entire salt range studied.

3.3.2 Salt Addition Titrations

In order to directly observe the effect of increased salt on the extent of the fractional
DNA saturation by protein, we performed salt addition titrations in the presence of KCl and
Kglutamate. DNA (1nM) was loaded past saturation with 5nM Klenow or 500nM Klentaq at low
salt concentration (5mM) and the salt concentration was then increased. A first qualitative look
confirms that the salt dependence of the protein bound DNA fraction is biphasic in the presence
of Kglutamate but not in the presence of KCl. As salt concentration is increased, the fraction of
bound DNA initially decreases sharply (Figure 14). This decrease is observed irrespective of the
anion present in solution. The decrease shows that the addition of salt displaces the proteins from
the DNA. At 0.5M, the displacement is almost complete in all instances, ~80-90% in Kglutamate
and ~95-100% in KCl (Figure 14). As salt concentration is increased past 0.5 M, however, a
second transition resulting in an increase in the fraction of bound DNA is observed. This second
transition is dependent on the nature of the anion present in solution (Figure 14). At 2.5 M
Kglutamate, almost 100% rebinding of the protein to DNA is observed. In contrast, addition of
KCl past 0.5M up to ~3.5 M (~1.5M for Klentaq) does not cause any significant rebinding. The
fraction of rebound DNA in increasing KCl concentrations was never observed to increase past
20% rebinding for Klenow while it remains at 0% for Klentaq (Figure 14).

A quantitative model describing the dependence of the DNA fractional saturation on salt
concentration was derived from the expression of the fractional saturation of DNA by protein
and the expression of the equilibrium association constant (Eq. 19, shown below). The fit to the
model yields thermodynamic parameters which can be compared to previously obtained
parameters (linear and non linear thermodynamic salt linkages).
Figure 14: Salt addition titrations of 5nM Klenow (panel A) and 500nM Klentaq (panel B). DNA was saturated with protein at low salt concentration. DNA binding was monitored as salt was titrated into the solution. Parameters obtained by non-linear fitting (Eq. 19) are displayed in Table 8.

\[
\theta = \frac{[PA_{\Delta \mu}^\text{salt}][K_A]}{[PA_{\Delta \mu}^\text{salt}][K_A] + \text{Salt}^{\Delta \mu_{\text{osm}}}[e^{\left(\frac{\alpha \text{[Salt]} + \beta}{55.5}\right)}]} \quad (\text{Eq. 19})
\]

Similarly to the non linear analysis of the extended salt linkage, this model also attempts to dissect the contributions from ion release and osmotically stressed water released. The model predicts that, at low salt, the ionic contribution is predominant yet increasingly unfavorable as
salt concentration is increased. On the other hand, the osmotic contribution, if present, is predominant at high salt and increasingly favorable as salt concentration increases. In order to account for the osmotic component, the water activity was expressed as a function of molar salt concentration by measuring the osmolarity of increasing salt concentration solutions as previously done for the non-linear extended salt linkage. The slope ($\alpha$) and intercept ($\beta$) were determined for both KCl and Kglutamate (Figure 13) and fixed in the salt addition fit (Figure 14). The fit yielded the parameter $i$, for the number of ions displaced by protein-DNA binding and the parameter $w$, for the number of salt induced osmotically stressed waters of hydration which are displaced by DNA binding.

However, these fits have several caveats that must be discussed prior to the interpretation of the obtained parameters. For instance, the non linear expression used in equation 19 proved to be less robust than the one used in extended salt linkage (Eq. 16). Indeed, the protein concentration used to saturate the DNA greatly affected the parameters obtained in the presence of Kglutamate. Upon increasing the Klenow concentration from 5 to 100nM, the displacement and rebinding transitions became undistinguishable. In other words, with 100nM Klenow, 80% of the DNA remained bound throughout the entire titration and, consequently, the quantitative parameters obtained by the fit were reduced more than 50%. The most distinguishable transitions obtained with 5nM Klenow in Kglutamate still resulted ~10 to 20% protein bound DNA throughout the entire titration in Kglutamate. In such conditions, we obtained a different number of ions released in KCl and Kglutamate with ~3 and ~4.1 respectively (Table 8). Due to the lack of complete displacement of the Klenow from DNA in Kglutamate, it is necessary to interpret these parameters with caution. In addition, the extended salt linkage analysis has shown that the number of linked ion release in KCl and Kglutamate are similar. Another caveat is the increasing random noise at high salt concentration. As salt concentration is increased above
Table 8: Salt addition of Klenow (5nM) and Klentaq (500nM) in KCl and Kglutamate. The fits yielded the ion released upon DNA binding (\(i\)) and the water released (\(w\)). \(w\) was not fit for in the presence of KCl.

<table>
<thead>
<tr>
<th></th>
<th>Klenow (5 nM)</th>
<th>Klentaq (500nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KGlu</td>
<td>(i=4.1 \pm 0.2)</td>
<td>(i=4.5 \pm 0.9)</td>
</tr>
<tr>
<td></td>
<td>(w=162.9 \pm 7.7)</td>
<td>(w=160.1 +/- 30.0)</td>
</tr>
<tr>
<td>KCl</td>
<td>(i=3.0 \pm 0.2)</td>
<td>(i=3.1 \pm 0.2)</td>
</tr>
<tr>
<td></td>
<td>(w ) (not fitted for)</td>
<td>(w ) (not fitted for)</td>
</tr>
</tbody>
</table>

1.5M, increased error in the anisotropic signal is observed in both the salt addition and background titrations. As a result, the background-subtracted normalized anisotropy signal displays high random noise beyond 1.5M salt, as shown for Klenow in KCl and Kglutamate (Figure 14A) and Klentaq in Kglutamate (Figure 14B). For Klenow in KCl, the random noise observed in the background-subtracted signal of the unbound DNA (after the displacement transition) varies between 5% and 15% of the total signal. Upon fitting with equation 19, the statistical fit to the noise forces a rebinding transition. Such transition happens beyond the solubility limit of chloride with a theoretical 50% rebinding at 5M and 100% rebinding at 7M. These values are experimentally inaccessible and the statistical fit obtained must also be interpreted with caution. We determined by salt addition that ~160 waters are released by Klenow-DNA binding in Kglutamate (Table 8). In KCl, the data was fit to a modified version of equation 19 which does not include an osmotic contribution (Figure 14A). The numbers of ions released (\(i\)) was found to be unaffected by the modification.

In comparison, the salt addition of Klentaq in Kglutamate yielded ~4.5 ions and ~170 waters released. Klentaq also displayed a protein dependence on the parameters obtained albeit differently. Indeed, in the presence of Kglutamate, the rebinding transition is observable at Klentaq concentrations above 100nM. In KCl, the fit to a model containing both an electrostatic
and osmotic component does not converge. The fit to a model containing only an electrostatic effect of KCl on DNA binding yielded ~3.1 ions released (Table 8).

3.4/ Nucleotide Incorporation

The characterization of the effect of salt, and in particular the physiologically relevant glutamate anion, on Klenow and Klentaq was extended to the functionality of the two proteins. A radioactive nucleotide incorporation assay was performed to determine the amount of nucleotide incorporated at several time points in the presence of Kglutamate (Figure 15, Table 9). The dependence of the steady state rate of incorporation on [KGlut] was then plotted together with the corresponding data in KCl in Figure 16.

Klentaq is enzymatically inactive at the temperature at which DNA binding studies were performed (25°C). Therefore, the nucleotide incorporation assays were performed at 35°C. Such temperature is near optimal for E.coli and Klenow but far from the optimal temperature for T.aquaticus and Klentaq. This difference in optimal temperature of enzymatic activity results in significant differences in total nucleotides incorporated by Klenow and Klentaq as observed in the assay. Indeed, under any solution condition, the amount of nucleotide incorporated by Klenow was determined to be at least one order of magnitude greater than that for Klentaq. In addition, the steady state rate of nucleotide incorporation is consistently greater in Klenow than in Klentaq. Since the parameters of interest are the effects of salt on relative nucleotide incorporation rates, these differences were deemed acceptable for initial comparisons. It is possible that salt dependence of Klentaq nucleotide incorporation at higher temperature may differ. Several observations can be made of the effect of increasing salt concentration and varying anionic salt types on nucleotide incorporation. First, as salt concentration increases, the total incorporation and the steady state rate of nucleotide incorporation generally decrease for both proteins. However, at low salt concentration (<0.2M), the enzymatic activity of Klenow is
Figure 15: Steady state nucleotide incorporation kinetic assay of Klenow and Klentaq with primed M13mp18 DNA. In the presence of Kglutamate at various concentrations, the amount of nucleotide incorporated was measured at various times (20, 40 and 60 min).
Table 8: Steady state nucleotide incorporation kinetics of Klenow and Klentaq with primed M13mp18 DNA. The amount of nucleotide incorporated at each time point and for each salt concentration is reported. The steady state rate is also listed.

<table>
<thead>
<tr>
<th>[KGl] M</th>
<th>Nucleotides incorporated 20 min (pmoles)</th>
<th>Nucleotides incorporated 40 min (pmoles)</th>
<th>Nucleotides incorporated 60 min (pmoles)</th>
<th>Steady state rate (pmoles/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klenow</td>
<td>0.1</td>
<td>1965.3 ± 32.9</td>
<td>2908.5 ± 97.4</td>
<td>3960.6 ± 79.7</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1529.5 ± 40.2</td>
<td>2624.2 ± 62.3</td>
<td>3844.8 ± 58.9</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>926.1 ± 61.7</td>
<td>1751.0 ± 112.2</td>
<td>2262.8 ± 167.1</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>724.3 ± 53.7</td>
<td>1247.1 ± 109.6</td>
<td>1927.3 ± 15.5</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>320.3 ± 10.4</td>
<td>472.6 ± 20.1</td>
<td>674.2 ± 19.4</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>175.3 ± 2.5</td>
<td>236.3 ± 0.5</td>
<td>316.8 ± 9.25</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>123.9 ± 2.1</td>
<td>142.5 ± 1.1</td>
<td>172.4 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>107.4 ± 6.5</td>
<td>128.8 ± 5.5</td>
<td>139.6 ± 4.5</td>
</tr>
<tr>
<td>Klentaq</td>
<td>0.05</td>
<td>155.9 ± 2.1</td>
<td>189.7 ± 4.2</td>
<td>214.2 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>138.9 ± 4.4</td>
<td>163.9 ± 1.4</td>
<td>177.5 ± 11.0</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>103.5 ± 0.5</td>
<td>120.7 ± 0.9</td>
<td>135.6 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>56.0 ± 0.5</td>
<td>106.5 ± 0.5</td>
<td>121.0 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>22.3 ± 0.3</td>
<td>29.1 ± 0.7</td>
<td>44.3 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>11.5 ± 0.3</td>
<td>15.6 ± 2.2</td>
<td>18.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>10.9 ± 0.6</td>
<td>16.5 ± 1.82</td>
<td>19.5 ± 1.7</td>
</tr>
</tbody>
</table>

Activated by an increase in salt concentration as seen in Figure 16A. This behavior is observed in both KCl and Kglutamate. A similar salt induced enhancement of enzymatic activity at low salt concentrations was previously reported in DNA polymerase III in both KCl and Kglutamate. In contrast, Klentaq does not display such salt induced enhancement of enzymatic activity (Figure 16B). Second, the substitution of glutamate for chloride has significant effects on the sensitivity of the enzymatic activity to increases in salt concentration. The decrease in steady state rate of polymerization in response to increased salt occurs over a much wider salt concentration range in glutamate than in chloride for both Klenow and Klentaq. Indeed, in KCl
Figure 16: Normalized steady state rate of Klenow (Panel A) and Klentaq (Panel B) nucleotide incorporation into primed M13mp18 DNA as a function of salt concentration. The data for Kglutamate and KCl is displayed. The KCl data was obtained from Hiromi Brown (unpublished data).
the steady state rate is reduced by half at ~250mM salt for Klenow and 100mM salt in Klentaq.
Upon substitution of glutamate for chloride, the halfway point is shifted to above 400mM salt for
Klenow and 300mM salt for Klentaq (Figure 16).

Finally, in the presence of glutamate, a low yet detectable level of nucleotide
incorporation was observable into very high salt concentrations (up to 1.6M salt). For Klentaq, a
steady state rate of nucleotide incorporation of 0.22 pmoles/min was observed at 1.5M
K-glutamate. In KCl, corresponding rates were observed in the 300 to 400mM salt concentration
range. Similarly, in Klenow, a steady state rate of 1.2 pmole/min was observed at 1.2M
K-glutamate. In KCl, the corresponding rates were observed at ~500mM salt. These observations
correlate well with the common use of glutamate as a compatible solute in various organisms
and the documented effect of glutamate on DNA binding and enzymatic activities. However, it is worth noting that the increase of potassium glutamate
concentrations beyond 1M did not result in an increase of the enzymatic activity in parallel to the
rebinding observed at high potassium glutamate. Rather, it just maintains a low level of
nucleotide incorporation that is not observed beyond 500mM in KCl.
CHAPTER 4: DISCUSSION

4.1 Thermodynamic Characterization of the Glutamate Effect on Klenow

In this study, the effect of salt concentration and type on DNA binding by Klenow and Klentaq were examined with particular emphasis on the effect of the physiologically relevant glutamate anion. In this section, we will discuss the characterization of the effect of replacing chloride by glutamate on the linear thermodynamic linkages (salt, pH and osmotic stress) and non-linear thermodynamic linkages (salt) of Klenow-DNA binding.

4.1.1 Linear Thermodynamic Linkages Analysis

- Klenow Displays Anion Specific Effect on $\Delta n_{\text{ions}}$

Klenow DNA polymerase displays the ‘glutamate effect’ on DNA binding and on the number of thermodynamically linked ions released, $\Delta n_{\text{ions}}$. At any salt concentration, we observed a significant increase in DNA binding affinity when chloride is substituted by glutamate as the anion in solution. Furthermore, $\Delta n_{\text{ions}}$ is reduced by more than 50% for Klenow in glutamate (~2 ions) versus chloride (~4.5 ions) (Table 1, Figure 6). As a consequence, Klenow displays low nanomolar DNA binding affinities into much higher salt concentrations in glutamate (800mM) than in chloride (500mM) and the $\Delta G_{1M}$ in glutamate is enhanced by 2.4 kcal/mol versus in chloride. The DNA binding affinity enhancement upon replacement of chloride by glutamate has been observed for a number of other *E.coli* nucleic acid binding proteins such as DNA polymerase III$^{93}$, RNA polymerase and various restriction enzymes$^{94}$, T7 RNA polymerase$^{96}$, recA$^{99}$, Rnase III$^{95}$ or the transcription termination factor Rho$^{97}$; $^{98}$. Furthermore, the effect of glutamate on the thermodynamic salt linkage of Klenow-DNA binding ($\Delta n_{\text{ions}}$) was similar to that previously characterized for Lac repressor (specific binding)$^{100}$, ssb protein$^{101}$ and IHF$^{102}$.

Other anions such as fluoride were also observed to decrease $\Delta n_{\text{ions}}$ to a similar extent in
Klenow. In the presence of fluoride, aspartate or D-glutamate, \( \Delta n_{\text{ions}} \) was found to be \( \sim 2 \) ions and within error from that of glutamate and the \( \Delta G^0_{1M} \) was determined to be similar to that in glutamate (Table 1 and Figure 6). The varying chemical nature of these anions suggests that the “glutamate effect” does not result from a strong specific site binding of glutamate to the protein but rather to differences in general anion-macromolecular surface chemistry and its effect on protein-DNA interactions. In addition, neither glutamate nor fluoride cause a significant differential shift in \( T_m \) of Klenow relative to chloride (Table 6, Figure 10) suggesting that coupled anion dependent stabilization of marginally stable Klenow does not play a role in the glutamate effect.

The origin of these anion specific effects on macromolecular processes remains unclear. In the case of glutamate, a general consensus emerging from the literature is that glutamate is further excluded from the vicinity of protein surfaces than chloride. Preferential interaction studies performed by Arakawa and Timasheff show that sodium glutamate is more excluded from lysozyme and BSA than sodium chloride\(^8^6;\,13^6\). The same studies show that glutamate is also significantly excluded from the vicinity of \( \beta \)-lactoglobulin and calf brain tubulin\(^8^6\). Courtenay et al have also observed an unfavorable preferential interaction of glutamate with BSA\(^1^0^5\). This difference in preferential interaction or exclusion of glutamate from the protein surface was proposed to be consistent with the increase in tubulin’s \( T_m \)\(^8^6\), as well as glutamate’s ability to counteract urea unfolding of FtsZ\(^8^8\) and glutaminyl-tRNA synthetase\(^8^7\). The relative exclusion of glutamate vs. chloride was proposed to explain the decrease in the thermodynamic salt linkage (\( \Delta n_{\text{ions}} \)) associated with DNA binding observed in several \( E.\,coli \) DNA binding systems. In other words, the anion specific decrease in \( \Delta n_{\text{ions}} \) (\( \Delta n_{\text{ions}} = \Delta n_{C^+} + \Delta n_{A^-} \)) has been interpreted in terms of a difference in the thermodynamic participation of anions \( \Delta n_{A^-} \).\(^1^0^0;\,1^1^1;\,1^1^2\).
This interpretation has led to the assumption that $\Delta n_A$ in glutamate or fluoride salts was close to zero and that $\Delta n_{\text{ions}}$ was predominantly due to the thermodynamic participation of cations $\Delta n_{C^+}$.[111;112]

Alternatively, the differences in preferential interactions or varying degrees of partitioning between the bulk and local domains of the solutions by different anions could also lead to anion specific osmotic stress of the water in the immediate vicinity of the protein surface.[111] Protein-DNA interaction perturbs the osmotically stressed hydration water at the binding interface and releases it into the bulk domain contributing an entropically favorable osmotic component. The favorable entropic contribution from water release increases as the salt concentration increases and the bulk thermodynamic water activity is decreased. Anion specific differences in hydration water resulting from differences in preferential interaction of the anions themselves could therefore explain the glutamate effect observed on $\Delta n_{\text{ions}}$ and $\Delta G_{1M}^o$. Prior to this dissertation, the studies on the glutamate effect on E. coli Lac repressor and IHF DNA interaction represent the most rigorous attempt to quantitatively dissect contributions that reflect the sum of ionic (both cation and anion) and osmotic behavior[100;102] of glutamate. However, while the narrow salt range often used to obtain linear salt linkage data allows the diagnosis of the glutamate effect on $\Delta n_{\text{ions}}$ and $\Delta G_{1M}^o$, such analysis is not suited to fully dissect and characterize the possible ionic and osmotic contributions. For that purpose, it is first necessary to observe and characterize the osmotic role of glutamate. Therefore, the osmotic stress technique was used to quantitatively characterize the effect of relative exclusion of anions (glutamate vs. chloride) on the number of thermodynamically linked water release $\Delta n_{H_2O}$.

- **Glutamate Also Affects $\Delta n_{H_2O}$**

  The number of thermodynamically linked waters released ($\Delta n_{H_2O}$) upon
Klenow-DNA binding were measured using the osmotic stress technique and reported in our study. Polyethylene glycol was used as a stressing solute to measure the change in hydration. In 500mM KCl, we report a Δn_{H2O} of ~479 waters in PEG 6000 Da and ~537 waters in Ficoll 70 kDa (Figure 9). The obtained results are within error of each other and yield an average of ~509 waters released. The similarity in the water released strengthens the assumption of the absence of direct osmolyte-macromolecular interactions which could interfere with the DNA binding equilibrium. The hydration change upon Klenow-DNA binding was also obtained in the presence of glutamate. In 800mM Kglutamate, Klenow-DNA binding yields ~151 waters in PEG6000 and ~136 waters in Ficoll 70 (Figure 9). The Δn_{H2O} obtained are within error and we report an average ~144 waters released in Kglutamate. The substitution of chloride by glutamate reveals a dramatic dependence of Δn_{H2O} on the anion in solution. Indeed, we report a decrease of ~350 waters (~70%) released when chloride is substituted by glutamate. These results constitute the first direct observation of anion specificity on the number of thermodynamically linked water release associated with DNA binding as measured by the osmotic stress technique. The dramatic difference in Δn_{H2O} highlights a significant difference in osmotic behavior of the respective anions towards DNA binding by Klenow and consequently highlights the difference in the ability of different anions to osmotically stress the hydration water (or local domain) of protein. It confirms the necessity to consider anion specific osmotic behaviors in the interpretation of anion specific effect on Δn_{ions}.

We have used various sizes of PEG in order to better characterize the glutamate effect on Δn_{H2O}. The relative negative preferential interaction of PEG with various proteins were observed to correlate with molecular weight of the polymer\textsuperscript{133,134} indicating that PEG is sterically excluded from protein surfaces. Therefore, upon utilizing increasing size of PEG from 200 Da to 6000 Da, an increase in Δn_{H2O} was expected as observed in Hexokinase\textsuperscript{137}. Indeed, we
determined that the $\Delta n_{H_2O}$ was highly dependent on PEG size in the presence of KCl. However, the PEG size dependence of $\Delta n_{H_2O}$ was found to be highly dependent on the anion present in solution. The effect of the size of the solute on $\Delta n_{H_2O}$ is highly attenuated by the presence of glutamate as opposed to chloride. In the presence of KCl, $\Delta n_{H_2O}$ is predominantly dependent on the osmotically stressed water of hydration resulting from the exclusion of PEG. Therefore, as the size of PEG is increased, it is sterically excluded further away and results in a significant increase of the $\Delta n_{H_2O}$. On the other hand, in the presence of Kglutamate, we observe that the $\Delta n_{H_2O}$ is much less dependent on the osmotic stress caused by the PEG.

A conceptual thermodynamic model based on anionic differences in preferential interaction (or exclusion) can explain the anion specific differences on $\Delta n_{H_2O}$ and its PEG size sensitivity (Figure 17). In this model, in the presence of both glutamate and PEG (panel A), the relative exclusion of glutamate results in multiple local domains or regions of water of hydration with varying degrees of osmotic stress. In the immediate vicinity of the protein surface within the binding cleft, region A is inaccessible to both glutamate and the stressing solute (PEG 6000 Da in Figure 17A). Based on this assumption, the activity of the water in region A, $a_w$(region A), is equal to 1 and is shown in dark grey in Figure 17. Further from the protein surface, region B is inaccessible only to the stressing solute (PEG 6000 Da). Under such assumption, the activity of water of region B (shown in light grey), $a_w$(region B) can be calculated from the measurement of osmolarity versus [Kglutamate] and equation 12. In 800mM Kglutamate, $a_w$(region B) is 0.9704. Finally, the bulk solution (colored white) contains both glutamate and PEG. The osmolarity of 10% PEG 6000 Da in the presence of 800mM Kglutamate was measured and $a_w$(bulk) was calculated to be 0.9607. The amplitude of the difference in thermodynamic activity of water is greatest between region A and the bulk solution. In addition, $a_w$(region A)-$a_w$(bulk) is
Figure 17: Molecular schematic of the thermodynamic model explaining the decrease in $\Delta n_{H2O}$ when chloride (panel B) is substituted by glutamate (Panel A). The range of increasing water activity is color coded from white (bulk solution) to dark grey.
significantly greater than \(a_w(\text{region B})-a_w(\text{bulk})\), and would therefore predominantly account for the thermodynamic water linkage, \(\Delta n_{H2O}\) in the presence of PEG 6000Da.

This model is also consistent with the PEG size dependence of \(\Delta n_{H2O}\). Upon increasing the size of PEG, the region B (light grey) is increased in volume, however the region A remains constant, limited by the extent of glutamate’s exclusion. The observations that the \(\Delta n_{H2O}\) is much less dependent on the size of PEG in the presence of glutamate and that it is decreased by 70% when chloride is replaced by glutamate (in PEG 6000 Da) strongly suggest that the stressing of region A would be the predominant contribution to the \(\Delta n_{H2O}\) in the presence of Kglutamate. It also suggests that chloride has little to no osmotic contribution to the Klenow-DNA equilibrium (region A is absent in Figure 17, panel B). Based on the assumption that the osmotic contribution arises from a preferential exclusion of a solute or anion from the vicinity of the protein (partitioning constant \(K_p < 1\)), the lack of osmotic contribution of chloride supports the assumption that chloride is not preferentially excluded from the protein surface or that it is distributed within the water of hydration as suggested for IHF\textsuperscript{102}.

The significant anionic specificity of the hydration change associated with Klenow-DNA binding provides evidence that the glutamate effect on \(\Delta n_{\text{ions}}\) and \(\Delta G^{°}_{1M}\) must be interpreted by including a glutamate effect on the \(\Delta n_{H2O}\). As salt concentration is dramatically increased, the water activity is significantly decreased and such anion specific osmotic behavior (differences in preferential interactions) could result in significant deviation of salt linkage plots from linearity in the presence of glutamate (or fluoride or any ion with a \(K_p < 1\)). In turn, these deviations would cause the apparent decreases in \(\Delta n_{\text{ions}}\) and \(\Delta G^{°}_{1M}\) as the anion is changed. This hypothesis is consistent with the fact that all systems that have displayed a glutamate effect on \(\Delta n_{\text{ions}}\) have been characterized in significantly high glutamate concentration (>0.5M)\textsuperscript{100; 101; 102}. In contrast,
the two studies that have reported the absence of glutamate effect on $\Delta n_{\text{ions}}$, were performed at low glutamate ($< 0.4$M$)_{103; 104}$. The next step, then, is to quantitatively link the $\Delta n_{\text{H}_2\text{O}}$ and $\Delta n_{\text{ions}}$ effects on Klenow-DNA binding in Kglutamate. This was achieved by extending the thermodynamic space of previous salt linkages into much higher salt concentration and by quantitatively characterizing the glutamate effect with respects to the participation of ions and water in the thermodynamic linkage.

4.1.2/ Non Linear Analysis of Extended Salt Concentration Ranges

- The Thermodynamic Salt Linkage at High [Kglutamate] Deviates from Linearity

Upon increasing the Kglutamate concentration, we observed that the DNA binding affinity of Klenow decreases from 200mM to 800mM Kglutamate, displays a minimum at 800mM Kglutamate and increases beyond 800mM up to 2M (Figure 11, 12 and Table 7). The increase in DNA binding affinity is such that the $K_d$ was determined to be more favorable at 2M than at 200mM Kglutamate. In other words, above 800mM Kglutamate, we observe a reversal of salt linkage resulting from the apparent increase of DNA binding affinity. Such reversed salt linkage occurred in the same salt concentration range as the reversed linkage observed in the study of the TATA binding protein from *Pyrococcus woesei* (*PwTBP*) by O’Brien *et al.*$^{107}$, albeit in glutamate. That system, and the Klenow and Klentaq results reported here constitute the only documentation of a reversal of salt linkage for any protein-DNA interaction. At such high salt concentration, the osmotic pressure induced by the salt overlaps the range probed by the osmotic stress techniques. It suggests that the observed effect of glutamate on $\Delta n_{\text{H}_2\text{O}}$ obtained by the osmotic stress technique is mirrored by a significant apparent enhancement of DNA binding by Klenow as Kglutamate is increased. A solution/molecular interpretation of the salt linkage up to 2M Kglutamate suggests that at low salt ($< 0.5$ Kglutamate), increasing concentrations of Kglutamate results in the gradual loss of the favorable contribution from counterion release. At
an intermediate salt concentration (0.5 to 1M Kglutamate), the water activity in the bulk solution is significantly decreased and the gain from the favorable contribution of osmotically stressed hydration water counteracts and balances the loss of the favorable counterion release. This behavior is due to entropically favorable release of waters that have been osmotically stressed by glutamate, and results in the leveling of the salt linkage. This leveling of the salt linkage seems to be the cause of the anion specific apparent decrease in $\Delta n_{\text{ions}}$ and $\Delta G^o_{1\text{M}}$ obtained by the traditional linear regressions of salt linkage over a narrow salt range. As the salt concentration is increased beyond 1M, the osmotic effects of glutamate on the equilibrium predominate over the ionic effects and results in the observable increase in DNA binding affinity.

The quantitative model used (Eq. 16) to describe the extended salt linkage takes into account both the ionic and osmotic contribution of Kglutamate towards DNA binding. The fit yields $\sim$5.3 ions and $\sim$196 waters released associated with DNA binding (See Table 7). The parameters obtained strongly validate the model used to explain the behavior of glutamate. First, the number of ions released is within error of the $\Delta n_{\text{ions}}$ in KCl (4.9 ions) obtained by linear regression of the salt linkage and much different than $\Delta n_{\text{ions}}$ in Kglutamate (2.1 ions) (See Table 7). In other words, the non-linear analysis of Klenow-DNA binding in Kglutamate restores the $\Delta n_{\text{ions}}$ value obtained in KCl, a salt believed to lack an osmotic effect on Klenow-DNA binding. This observation weakens somewhat the interpretation that the glutamate effect results from a decrease in the anionic contribution ($\Delta n_{\text{A}^-}$) to the linked ion release. So it would seem to suggest that the non-linear fit can quantitatively dissect the electrostatic contributions from the osmotic contribution. Second, the number of osmotically stressed waters released is also similar to that obtained by the osmotic stress technique, although not within error. Indeed, $\Delta n_{\text{H2O}}$ in the presence of Kglutamate was determined to be $\sim$151 waters. The quantitative agreement between these two parameters confirms the interpretation that the significant glutamate effect on $\Delta n_{\text{H2O}}$...
obtained by the osmotic stress technique and the reversal in salt linkage observed in the extension of salt linkage into high salt are both due to the same osmotic effect of glutamate on Klenow-DNA binding. Finally, the non-linear analysis also restores the value of the salt independent reference free energy of binding parameter ($\Delta G_{\text{ref}}^o = -6.2 \text{ kcal/mol}$) to that observed in the linear regression in the presence of KCl ($\Delta G_{1\text{M(Cl)}}^o = -6.6 \text{ kcal/mol}$ vs. $\Delta G_{1\text{M(Glu)}}^o = -9.0 \text{ kcal/mol}$) (Table 7). Also, it is worth noting that the addition of the energetic contributions of the ionic and osmotic behaviors of Kglutamate is always favorable throughout the entire concentration range. In other words, at any [Kglutamate], $\Delta G_{\text{obs}}^o$ is always more favorable than $\Delta G_{\text{ref}}^o$. The weakest DNA binding affinity occurs at ~800mM [Kglutamate], still the $\Delta G_{\text{obs}}^o$ is equal to ~1.6 fold $\Delta G_{\text{ref}}^o$ obtained from the fit.

These applicable correctabilities of the parameters obtained through the standard linear regression analysis relative to the non-linear model developed is a strong indication of the validity of the model and strengthens the confidence in the parameters obtained. It further confirms that the apparent shift in $\Delta n_{\text{ions}}$ in the presence of glutamate relative to chloride observed in the linear analysis is not representative of a difference in stochiometry of the counterion release or a difference in the stochiometry of anion release. The linear regression to fit the salt linkage within a limited salt range must be viewed with caution as it may not accurately represent all the thermodynamic contributions and molecular events involved in the process.

*Direct Visualization of the Glutamate Effect at High Salt Using Salt Addition Titrations*

Using salt addition assays, we have been able to monitor the fractional saturation of DNA by Klenow and observe the effect of high salt concentration on Klenow-DNA binding. The qualitative comparison of the salt addition titrations of Klenow by KCl and Kglutamate suggests
that there isn’t any anion specific effect at low salt (<0.5 M) (Figure 14). However, upon increasing the salt concentration past 0.5M, the two anionic salts have different effects on the Klenow-DNA interaction. Potassium glutamate induces the rebinding of the protein which had been previously displaced. The salt addition titration of Klenow in Kglutamate provides a striking direct visualization of rebinding at high salt concentrations due to increased affinity. In contrast, potassium chloride does not induce rebinding and the protein remains displaced (Figure 14). It strengthens the observation that chloride has little to no favorable osmotic effect on Klenow-DNA binding. Chloride is solvated and is within the water of hydration of the protein to a similar extent as within the bulk water and results in little osmotic stress of the hydration water perturbed by DNA binding.

The salt addition titrations also are in good agreement with previous results in highlighting that the anion specific effect of glutamate vs. chloride occurs mostly in a high salt concentration range. However, the quantitative analysis of the salt addition titrations proved to be more difficult than that of the non-linear analysis of extended salt linkages. For instance, the statistical fit seemed to lack robustness in its dependence on Klenow concentration. The Klenow concentration had a significant effect on the resolution of the displacement and rebinding transitions. As a consequence, the parameters derived from the fit were highly dependent on protein concentration. At 5nM Klenow, the number of ions released was found to be different in Kglutamate and KCl (4.1 and 3.0 respectively) (Table 8). The non-linear linkage analysis, however, indicated that this parameter should be anion independent. The statistical fit also proved to be sensitive to random noise in the signal at high salt concentration (beyond 1.5M), most significantly in KCl. Whereas non-linear linkage suggests a lack of osmotic effect of chloride on Klenow-DNA binding, the statistical fit to the random noise in the signal at high KCl concentration suggested that ~68 waters are osmotically stressed by KCl. However, this

[80]
parameter results from a transition that happens beyond the solubility limit of KCl and therefore is experimentally inaccessible in Figure 14. Although this suggest that the fitted $\Delta n_w$ in the presence of KCl is due to random noise, previous studies have shown that chloride can be slightly excluded from protein surfaces\textsuperscript{136,138}. Furthermore, an osmotic contribution in a chloride salt was characterized by non-linear analysis of salt linkage in high [Cl$^-$] in $p_w$TBP\textsuperscript{107}. It is therefore not possible to completely rule out a minor osmotic contribution of chloride on Klenow-DNA binding. In the presence of Kglutamate, the rebinding transition is entirely experimentally inaccessible (Figure 14). Consequently, it was derived that $\sim$160 waters were osmotically stressed by glutamate (Table 8). This parameter is within error of $\Delta n_{H_2O}$ and in good agreement with the same parameter obtained by non-linear analysis of the salt linkage.

4.1.3 The Glutamate Effect in Klenow Is Predominantly an Osmotic Effect

The linear regression of salt and water thermodynamic linkages along with the non-linear analysis of extended salt linkage and salt addition titrations has allowed us to establish that the traditionally characterized glutamate effect on DNA binding is due to a significant osmotic role of glutamate on Klenow DNA binding. The traditional thermodynamic signature of the ‘glutamate effect’, the decrease in $\Delta n_{\text{ions}}$, is not indicative of anion specific differences in the stochiometric participation of anions ($\Delta n_{A^-}$) but rather from an osmotic contribution that acts in the opposite direction from the salt effect. Indeed, the extension of the salt linkage indicated that the net number of linked ions released in KCl and Kglutamate are similar. On the other hand, a new thermodynamic signature of the glutamate effect has been shown to be a dramatic effect on the thermodynamically linked net water release, as quantitatively characterized by the osmotic stress technique and the extended salt linkage. Qualitatively, this results in an apparent reversal of salt linkage at high salt and a rebinding transition in salt addition titrations. Such observations
are consistent with the hypothesis that glutamate is preferentially excluded from protein surfaces relative to chloride\textsuperscript{86; 105}. However, it suggests that the exclusion results mostly in a favorable energetic contribution from the release of osmotically stressed water of hydration rather than in a difference in the thermodynamic participation of ions. It is a solute induced solvent effect rather than a solute effect on DNA binding.

The DNA binding enhancement at high potassium glutamate which results from the osmotic role of glutamate may explain the intracellular accumulation of potassium and glutamate during osmoadaptation of \textit{E.coli} along with other osmolytes and/or osmoadaptation strategies\textsuperscript{59; 60; 64; 65; 66; 67; 68; 69; 70; 71; 82; 139}. It also complements the numerous studies of the glutamate effect on macromolecular processes in other \textit{E.coli} DNA binding systems\textsuperscript{93; 94; 95; 96; 97; 98; 99; 100; 101; 102}. However, some studies have reported cases of protein dependence of solute-protein chemistry. Indeed, Arakawa \textit{et al} have suggested that the electrostatics of the protein surface can affect the preferential interaction coefficient\textsuperscript{86; 140}. The study of DNA binding by \textit{PwTBP} was most indicative of the importance of the protein surface towards solute-protein chemistry and its consequence on the thermodynamics of protein DNA interaction. Contrary to all other systems previously studied, the binding affinity of \textit{PwTBP} was shown to increase with increasing chloride salt concentrations\textsuperscript{107}. The halophilic behavior was successfully attributed to carboxylic residues near the protein-DNA interface associated with cation uptake upon DNA binding. The mutation of four residues was shown to reverse the halophilic behavior of \textit{PwTBP} completely to a more traditional mesophilic behavior\textsuperscript{108; 109}. Such protein specificity highlights the need to characterize the glutamate effect on Klentaq DNA-binding.

\section*{4.2 Klenow vs. Klentaq}

\subsection*{4.2.1 The Glutamate Effect in Klentaq}

The growth of \textit{T.aquaticus} was shown to be inhibited at low salt concentrations by Brock
and Freeze. Sodium chloride concentrations of 1~2% inhibit growth of the thermophilic bacteria. For that reason, we have characterized the glutamate effect on Klentaq DNA binding and compared it to Klenow’s to examine if differences in the glutamate effect on the thermodynamics of DNA binding parallel the accumulation of glutamate in *E.coli* and its enhanced osmoadaptation relative to *T.aquaticus*. The putative differences in the glutamate effect on the thermodynamics of DNA binding by the two proteins could indicate protein specific adaptations that complement or take advantage of the thermodynamic properties of glutamate towards macromolecular processes in order to achieve better osmoadaptation.

Initially, the linear regression analysis of salt linkage displayed a somewhat different effect of glutamate on Klentaq than on Klenow DNA binding. Indeed, in the presence of glutamate, fluoride or aspartate, the thermodynamically linked ions released by Klentaq-DNA binding was found to be similar to that in the presence of chloride and acetate (~ 3.5 ions) (Table 1). In contrast to Klenow, the glutamate effect on Klentaq’s Δ*n* ions appeared insignificant and the effect on Δ*G*°$_{1M}$ was much decreased. However, further investigation of hydration changes along with the non-linear analysis of salt linkage and the salt addition titration revealed that Klentaq and Klenow’s thermodynamic properties with respect to all solution components are very similar. It was determined that the substitution of chloride by glutamate reduces the Δ*n*$_{H2O}$ associated with Klentaq DNA binding by ~70% from ~520 to ~170 thermodynamically linked water released (Figure 9). The qualitative and quantitative analysis of the extension of the salt linkage and the salt addition titration of Klentaq-DNA binding show that at low salt (<0.5M), the favorable entropic contribution from counterion release is predominant yet progressively decreases as [Kglutamate] is increased. It is overcome by an increasingly favorable osmotic effect as [Kglutamate] is increased beyond 1M in a qualitatively and quantitatively similar manner as with Klenow.
4.2.2 Implication of the Salt Range Studied

The results observed in the case of Klentaq confirm that the linear regression analysis of the salt linkage must be performed and interpreted with caution. The anion nature and salt concentration range are critical for the accurate interpretation of the obtained parameters. Klentaq-DNA binding was initially measured in the range from 0.05M to 0.375M (Figure 3, -3<ln[Salt]<-1). In this range, the energetic contribution from the net linked ion release is predominant as shown by salt addition and confirmed by the extended salt linkage. As a consequence, the differences in the $\Delta n_{\text{ions}}$ obtained in that salt range by linear regression do not show significant differences upon changing the anion. They are strictly indicative of the energetic consequences of the release of counterions and cannot be used to diagnose or investigate the glutamate effect which is predominantly an osmotic effect at high osmotic pressure. The alpha subunit of RNA polymerase and cAMP receptor protein also display a lack of anion specificity on $\Delta n_{\text{ions}}$ obtained linearly at low salt concentration (<0.4M). On the other hand, initially, Klenow-DNA binding was measured from 0.2 to 0.5M in KCl and up to 0.8M in Kglutamate for the standard linear analysis of salt linkage. In this range, the osmotic contribution of glutamate is significant enough to distort to $\Delta n_{\text{ions}}$ obtained by linear regression but not enough for an accurate non-linear quantitative analysis. It is therefore necessary to cautiously interpret and/or discuss the data obtained through the analysis of a salt linkage plot performed on a narrow salt range, most importantly if the salt exhibits an osmotic contribution towards the macromolecular process. On the other hand, non-linear analysis over an extended salt range is more appropriate for accurate dissection of the thermodynamic participation of all components. The increase in the salt range studied allows a more complete characterization of the nature of the effect of ionic solutes on macromolecular processes. As such, it is also more suitable for the study and interpretation of the thermodynamic origins of molecular halophilic adaptations.
4.2.3 Klenow and Klentaq Display Overall Similarity in Thermodynamics with Respect to Solution Components

The linear and non-linear linkage studies allow the comparison of the thermodynamics of DNA binding by Klenow and Klentaq with regard to salt, pH and solvent effects. In particular, the non-linear extended salt linkages in Kglutamate allow an effective comparison of the glutamate effect on the thermodynamics of DNA-binding by these two proteins. Overall, it was determined that Klenow and Klentaq display very similar thermodynamics of binding over the extended Kglutamate concentrations examined. The similarities and differences are discussed in this section.

A first similarity is observed in the lack of anion dependence of the ionic contribution to the thermodynamics of DNA binding. The linked ion release obtained in KCl by linear regression is similar to the linked ion release obtained by non-linear analysis in Kglutamate for both proteins. It suggests that the relative exclusion of glutamate vs. chloride does not have any consequences on the thermodynamic participation of anion release ($\Delta n_{A^{-}}$). This observation must be interpreted in light of the recent characterization of a salt dependent enthalpy of binding for IHF and ssb DNA binding. While the thermodynamic linkage of salt on DNA binding was originally thought to be due to an entropic contribution of the release of condensed counterions from the DNA\textsuperscript{19}, recent studies\textsuperscript{102; 106} have characterized a salt dependent enthalpy of DNA binding suggesting that the nature of anion-protein surface interactions must be reconsidered. These studies have described a highly salt dependent enthalpy of DNA binding and in particular an anion specific effect on the $\Delta H_{\text{obs}}$ dependence on salt concentration that mirrors anion specific effects on the linked ion release. The dependence of the binding enthalpy of IHF on salt concentration has been shown to be greater in chloride salts than in fluoride or glutamate salt\textsuperscript{102}. Similarly, the linked ion release of ssb upon binding DNA was shown to be highly temperature
dependent in NaCl but not in NaF which resulted in a $\Delta H_{\text{obs}}$ dependent on the [NaCl] but not [NaF]\textsuperscript{106}. Although there are differing observations\textsuperscript{108} and interpretations\textsuperscript{141} of the salt dependence of the enthalpy of binding, the results from IHF and ssb suggested chloride might form direct dehydrated interactions with these proteins. However, a previous study has shown that the linked ion release of full length Taq polymerase in KCl remains largely unaffected at 60\textdegree C compared to 25\textdegree C\textsuperscript{113}. Furthermore, proton linkages in Klentaq display little dependence on the anionic species present in solution (Figure 7, Table 2). Thorough measurements must be performed, in particular for Klenow which displays anion specific proton linkage. However, the preliminary information seems to suggest the absence of direct dehydrated anion-protein interactions in Klentaq. Furthermore, anion specificity towards forming such dehydrated ionic interactions should display a dramatic anion specific effect on the linked ion release. However, in Klenow and Klentaq, the similarity in the linked ion release obtained linearly in KCl and non-linearly in Kglutamate does not support such hypothesis. On the contrary, it suggests that glutamate and chloride contribute insignificantly or very similarly to the $\Delta n_{\text{ions}}$ of their respective potassium salts.

Klenow and Klentaq DNA binding do not result in significantly large differences in thermodynamically linked ion release. Klenow results in 1 to 1.5 higher linked ion release independent of the nature of the anion. In KCl, the difference in $\Delta n_{\text{ions}}$ was determined to be about 1.5 ions whereas in Kglutamate, the difference in ion release was determined to be 1.1 ions by non-linear analysis. The difference in ion release by the two proteins reflects on the change in DNA binding affinity at low salt. As [Kglutamate] is increased from 200mM to 500mM, the extrapolated $K_d$ of Klentaq increases ~9.8 fold while that of Klenow increases ~14.9 fold. Interestingly, this difference indicates that at low salt the salt sensitivity of Klenow is higher than that of Klentaq. It is worth emphasizing the reported differences do not reveal a dramatically
different behavior between Klenow vs. Klentaq with regard to the ion release contribution towards DNA binding. The quantitative differences observed could be explained by a higher number of electrostatic contacts between Klenow and DNA than Klentaq to the same nucleic acid fragment. A possible explanation would involve the disruption of salt bridges in Klentaq upon DNA binding. Such phenomenon has been observed and characterized by a lowered linked ion release than predicted from the number of electrostatic contact between the protein and DNA. This hypothesis could be indirectly supported by the calculated electrostatic potential maps and proton linkage data. The difference in the positive $\Delta \text{ASA}$ upon binding by Klenow and Klentaq, along with the difference in anion specific behavior of the proton linkage of Klenow vs. Klentaq could be indirectly reflecting the involvement of disruption of salt bridges in Klentaq.

Klenow and Klentaq DNA binding also display similarity in the hydration changes and the extent of the osmotic contribution of glutamate effect. The PEG molecular weight dependence of DNA binding by Klenow and Klentaq are practically identical. The hydration change determined by the osmotic stress technique in the presence of chloride and PEG 6000 Da are similar and within error, with ~500 waters released from the interface upon binding. These similarities reflect the overall structural similarity of Klenow and Klentaq and in particular of the dimension of the binding cleft from which PEG 6000 Da is excluded. It is worth noting that Klentaq does not possess a functioning proofreading domain and always binds in the polymerase active site whereas Klenow, which has a functioning proofreading ability, can bind in either active site. Recent studies have suggested that the absence of added Mg$^{2+}$ tends to shift the partitioning towards the polymerase domain. Although added Mg$^{2+}$ was not included in the binding buffer to maximize the partition of the primer strand to the polymerase active site, the measurements of the extent of partitioning in Klenow were not performed. Therefore, at worst,
the similarities in Δ\(n_{\text{H2O}}\) suggest that if there is some partitioning into the proofreading active site by the primer strand, it is not detectable by the osmotic stress technique.

The similarities in the hydration changes associated with Klenow and Klentaq DNA binding extend to the consequences of replacing the anion in solution on the Δ\(n_{\text{H2O}}\). The substitution of glutamate for chloride decreases the Δ\(n_{\text{H2O}}\) about 70% to ~150 to 200 waters for both proteins. Furthermore, the similarity in the osmotic contribution of glutamate on DNA binding by the two proteins was confirmed qualitatively and quantitatively by extension of salt linkage and salt addition titrations. The glutamate anion contributes an osmotic effect on DNA binding by both Klenow and Klentaq resulting from the displacement of ~150-200 osmotically stressed waters. Thus, it suggest that the preferential exclusion (or partitioning) of glutamate from Klenow and Klentaq’s DNA binding interface are very similar.

In addition, surface area calculations have indicated similar extents of burial of surface upon DNA binding. Indeed, previous studies\(^{56; 57; 144}\) have reported that 2740 Å\(^2\) for Klenow and 2530 Å\(^2\) Klentaq are buried from the protein and DNA equally upon DNA binding. The reported area represents the sum of the ΔASA from both the protein and DNA, and the contributions from the protein surface and the DNA surface were also reported to be equal. It is to be noted that, in the case of Klenow, the co-crystal structure (1KLN\(^{35}\)) displays Klenow in the editing mode, not the polymerase mode. Furthermore, the calculations are based on co-crystal structures and do not take into account possible coupled processes that could result in burial of available surface area upon DNA binding. Nevertheless, using the assumption that one water molecule occupies 7-10 Å\(^2\), the number of waters displaced from the first layer of hydration water on the proteins would amount to ~137 to 195 waters in Klenow and ~127 to 181 waters in Klentaq. The thermodynamically linked waters released in the presence of glutamate which are reported in this
work are within these calculated values. Under the assumption that glutamate’s preferential exclusion only affects the protein surface and not the DNA surface, this structural calculation provides corroborating evidence that the first layer of hydration water of the proteins is inaccessible to glutamate in both Klenow and Klentaq. This glutamate-inaccessible first layer of water corresponds to the region A in Figure 17 and would result to the favorable osmotic effect when this water is displaced upon DNA binding in the presence of glutamate.

The complete dissection of the thermodynamic participation of ions and water and the characterization of the glutamate effect towards DNA binding by the homologous large fragments of DNA Pol I from salt sensitive *T.aquaticus* and moderately salt tolerant *E.coli* reveals that the homologous proteins behave very similarly with regards to these solution components and that both display a glutamate effect which was quantitatively equivalent. The overall similarity is most strikingly illustrated by the extension of salt linkage in Kglutamate up to 2.5M (Figure 12). Over the entire salt range, the extended salt linkage plots of Klenow and Klentaq display similar curvatures and the fold difference in DNA binding affinity is relatively constant with \(-2.4\text{kcal/mol}<\Delta\Delta G^\circ_{\text{obs}}(\text{Kln-Ktq})<-3.4\text{ kcal/mol}\). The glutamate effect on the thermodynamics of Klenow-DNA binding correlates well with the moderate tolerance of salt by *E.coli* achieved through the primary accumulation of intracellular glutamate. However, its quasi similar effect on the thermodynamics of Klentaq-DNA binding suggest that the glutamate effect does not result from a molecular halophilic adaptation strategy (such as that of *PwTBP*) in *E.coli* proteins which would modulate glutamate-protein surface chemistry in order to induce or enhance the favorable contributions of the glutamate effect and therefore improve osmoadaptation. While our pair of homologous proteins does not display such molecular halophilic adaptation, it is necessary to point that *E.coli* is not a halophile and that its natural
habitat might not subject it to the extremes that might induce such dramatic adaptive strategies at the molecular level.

4.2.4 ΔΔGo(Kln-Ktq), a Molecular Level Adaptation for Osmoadaptation

While the ΔΔGo(Kln-Ktq) is relatively constant, its amplitude is significantly large (See Figure 12). The ΔΔGo(Kln-Ktq) is least favorable at ~800mM Kglutamate with a value of -2.4 kcal/mol. As the Kglutamate concentration is varied away from 800mM, the ΔΔGo(Kln-Ktq) growth slightly more favorable up to -3.4 kcal/mol at 2.5M Kglutamate and -2.6 kcal/mol at 100mM Kglutamate. The large ΔΔGo(Kln-Ktq) could be very significant in the moderate osmoadaptation of E.coli vs. T.aquaticus. The much more favorable free energy of binding of Klenow allows it to maintain very low nanomolar binding affinity throughout the entire Kglutamate concentration range. The maximal Kd was determined to be ~30nM at ~800mM Kglutamate. On the other hand, comparable DNA binding affinity occurs at around 100 to 150mM Kglutamate for Klentaq and at 500mM Kglutamate, Klentaq-DNA binding is in the micromolar Kd range. A further look into the quantitative parameters derived from the non-linear analysis can provide some primary information with regards to the origins of the ΔΔGo(Kln-Ktq) and ΔΔGo(Klenow) values obtained non-linearly in Kglutamate (-4.8 kcal/mol in Klentaq, -6.2 kcal/mol in Klenow) (Table 7) matched the ΔGo(KCl) obtained linearly in KCl (-5.3 kcal/mol in Klentaq, -7.3 kcal/mol in Klenow) (Table 1). Furthermore, in Klentaq as in Klenow, we observe that the least favorable ΔGo(Kglutamate) at ~800mM [Kglutamate] is 1.6 fold the ΔGo(KCl). As a consequence, the least favorable ΔΔGo(Kglutamate) (~2.4 kcal/mol at ~800mM Kglutamate) is 1.6 fold the salt independent ΔΔGo(KCl). This observation suggests that the large ΔΔGo(Kglutamate) predominantly originates in differences in the salt independent ΔGo(KCl). It would further suggest that the macromolecular differences or adaptations that result in the much more favorable DNA binding
by Klenow throughout the entire salt range primarily affect the intrinsic binding of Klenow DNA binding relative Klentaq rather than the thermodynamic participation of ions and water towards the binding of both proteins to DNA.

The $\Delta \Delta G^0_{\text{obs}}(\text{Kln-Ktq})$ could be indicative of a macromolecular adaptation that together with the accumulation of intracellular glutamate allows high affinity DNA binding by *E.coli* DNA binding proteins to persist into higher salt concentrations. Little is known about the changes in intracellular environment of *T.aquaticus* in response to external osmotic stress. *T.aquaticus* lacks the enzymatic machinery to synthesize the osmolytes found in the closely related halotolerant *T.thermophilus* suggesting that *T.aquaticus*’ salt sensitivity is due to its inability to accumulate compatible solutes to counteract changes in turgor pressure upon osmotic shock. On the other hand, *E.coli* counteracts the increases in turgor pressure through the sequential accumulation of potassium followed by glutamate. Consequently, *E.coli* DNA binding proteins could have adapted to bind intrinsically with higher affinity in order for DNA binding to persist upon the initial increase in potassium. The subsequent accumulation of glutamate would then allow DNA binding to persist into much further salt concentration through its affinity enhancing osmotic effect on DNA binding. Such adaptation could reveal a different thermodynamic approach to moderate halophilic adaptation which involves an increase in the intrinsic DNA binding as opposed to the dramatic halophilic adaptation of *PwTBP* which modulates the DNA binding affinity as salt concentration is changed. However, the lack of direct comparative studies does not allow us to generalize such an adaptive molecular strategy for other *E.coli* proteins.
4.3 The Effect of Glutamate on the Catalytic Activity

4.3.1 The Effect of Replacing Chloride by Glutamate on the Persistence of Nucleotide Incorporation into High Salt Concentration

In order to understand the physiological relevance of the glutamate effect on DNA binding, the consequences of the substitution of chloride by glutamate on the enzymatic activity of both proteins was investigated. Several studies have investigated the effect of glutamate on the enzymatic activity of DNA binding enzymes from *E.coli* such as DNA polymerase III\(^93\), T7 RNA polymerase\(^96\), *E.coli* Rep protein\(^135\), transcription termination factor Rho\(^97;\ 98\), RNA polymerase and various restriction enzymes\(^94\) and recA\(^99\). In the systems where the salt dependence of enzymatic activity was performed, it was observed that an increase in salt concentration decreases the activity\(^93;\ 94;\ 96;\ 99;\ 135\). Furthermore, in some cases, the decrease in enzymatic activity was linked to the effect of salt on the protein-DNA interaction\(^93;\ 94;\ 96\). The substitution of chloride by glutamate systematically resulted in an upshift in the salt range at which enzymatic activity was observed. The shift was suggested to be due to the enhanced DNA binding in the presence of glutamate. However, in *E.coli* recA, it was determined that 1.5M sodium glutamate did not dissociate the complex yet ATPase activity is significantly reduced or abolished.

In Klenow and Klentaq, it was determined that the enzymatic activity as measured by the steady state rate of incorporation was decreased as salt concentration is increased. The substitution of chloride by glutamate increases the persistence of enzymatic activity into high salt as suggested by the shift in halfway points (Figure 16). It does not abolish the salt dependence of enzymatic activity. The comparison of the salt dependence of nucleotide incorporation and the salt addition titrations allows one to correlate visually DNA binding and nucleotide incorporation (Figure 18). It was observed that the salt range at which DNA is displaced from the proteins
Figure 58: Overlapping plots of the steady state rate and fractional saturation of Klenow (panel A) and Klentaq (panel B) in KCl (red) and Kglutamate (blue). The steady state rate was obtained from the nucleotide incorporation assay performed with 1uM protein. The fractional saturation was obtained from salt addition titrations with 5nM Klenow and 500nM Klentaq, and then fit to equation 19.
overlaps with the salt range at which the steady state rate decreases. This qualitative description suggests that the decrease in the steady state rate as salt concentration is increased results from the decreased DNA binding. It corroborates previous studies that suggested that salt effects on enzymatic activity of nucleic acid modifying enzymes are primarily due to the salt effects on DNA binding. The increased persistence of nucleotide incorporation into high salt in the presence of potassium glutamate is therefore due to the enhanced DNA binding in the presence of glutamate. It highlights the physiological relevance of the glutamate effect on DNA binding in maintaining critical functionality such as DNA replication and gene expression during osmotic shock.

4.3.2 Nucleotide Incorporation Is not Enhanced Above 1M Kglutamate

In both Klenow and Klentaq, low yet detectable levels of nucleotide incorporation have been observed as high as 1.5M Kglutamate. DNA binding studies have revealed that at such high concentrations of potassium glutamate, the osmotic contribution of glutamate is predominant and results in an increase in DNA binding affinity as the concentration is increased. However, the high [Kglutamate] induced DNA binding does not result in an increase in nucleotide incorporation. This phenomenon was also observed with the DNA dependent ATPase activity of recA at 1.5M99. The lack of correlation between DNA binding and enzymatic activity is not unique to high potassium glutamate. Indeed, Klentaq has been shown to bind primed template DNA with high affinity down to 5°C yet it does not perform any catalytic activity below 30°C.

The physiological relevance of this behavior in high potassium glutamate concentration must be addressed. In response to an external increase in osmotic pressure, it has been demonstrated that potassium then glutamate are accumulated intracellularly65; 66; 67; 68; 69; 71. This initial response is followed by the progressive switching to non ionic compatible solutes such as proline or betaine derivatives if present in the extracellular environment62; 64; 65; 71; 139. In the
absence of such compatible solutes, *E. coli* was found to partially replace potassium and glutamate by synthesizing trehalose\textsuperscript{65; 67; 70; 71}. Upon replacement of potassium and glutamate, *E. coli* was observed to grow and perform cellular activities into higher salt external salt concentrations. *E. coli* grown in minimal media and lacking the ability to synthesize trehalose were inhibited by 3 to 4% NaCl in the media\textsuperscript{69}. In such conditions, different studies have quantitated the intracellular glutamate concentration at around \(~0.3\) \textsuperscript{66 to 0.4\textsuperscript{67} molal. In the presence of non ionic compatible solutes, growth and cellular functionalities were observed up to 5 to 6% NaCl\textsuperscript{64; 65; 139}. Physiologically, Klenow and other *E. coli* DNA binding enzymes will not encounter such high concentration of potassium glutamate as experimentally probed.

Furthermore, potassium and glutamate seem to be insufficient to restore metabolic activity and growth at high osmolarity (beyond 3-4% or \(~1-1.5\ Osm). Rather it accumulates other compatible solutes. Other organism such as *Methanococcus thermolithotrophicus*\textsuperscript{78} or *Halobacillus halophilus*\textsuperscript{79} have also been described to switch osmolyte strategies away from glutamate at high concentration. These observations could reflect the inhibitory effect of high glutamate concentrations on enzymatic activity while enhancing DNA binding. So, while we have shown that the entropically favorable of release of osmotically stressed water leads to the formation of highly stable protein-DNA complexes, it remains to be determined why such complex would not be conducive to efficient nucleotide incorporation. Among possible models to explain these observations is a possible inhibition of efficient translocation at such high [Kglutamate].
REFERENCES


denaturation of *Thermus aquaticus* and *Escherichia coli* type 1 DNA polymerases.


and thermodynamics of Klenow polymerase binding to primed-template DNA. *Biophys J* **90**, 1739-51.

57. Datta, K. & LiCata, V. J. (2003). Thermodynamics of the binding of *Thermus aquaticus*


60. Empadinhas, N. & da Costa, M. S. (2008). Osmoadaptation mechanisms in prokaryotes:


of proline and K+ on the stimulation of cellular activities in *Escherichia coli* K-12 under


64. Cayley, S. & Record, M. T., Jr. (2003). Roles of cytoplasmic osmolytes, water, and
crowding in the response of *Escherichia coli* to osmotic stress: biophysical basis of

*Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and

Record, M. T., Jr. (1987). Variability of the intracellular ionic environment of
*Escherichia coli*. Differences between in vitro and in vivo effects of ion concentrations


[103]


APPENDIX A: COMPLEMENTARY DATA

Table 9 lists the equilibrium dissociation constants (K_d) of Klenow-DNA binding at different pH values. The tables present data obtained at two different KCl concentration (300mM and 500mM). In 500mM KCl, the data listed represents the K_d prior to adjustment. In contrast, the values listed in Table 2 have been adjusted using the K_d obtained at pH 7.9 with 500mM KCl in Table 1. The thermodynamic proton linkage remained unchanged by the adjustment. Also, Klenow’s proton linkage was obtained at 300mM KCl using protein from the same preparation. It was done in order to obtain low nanomolar K_d to increase the confidence in the thermodynamic proton linkage. The value of the proton linkage was found to remain unchanged at 300mM KCl.

Table 9: Equilibrium dissociation constants (K_d) of the interaction Klenow with primed-template at varying pH. The dissociation constants were obtained in the presence of 300mM or 500mM KCl. The resulting proton linkage (ΔnH+) is listed. The K_d in 500mM KCl represent the raw, unadjusted K_d value obtained. The values listed in Table 2 were adjusted for the standard value at pH 7.9, 500mM KCl.

<table>
<thead>
<tr>
<th>Salt type</th>
<th>Klenow (300mM salt)</th>
<th>Klenow (500mM salt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>K_d</td>
</tr>
<tr>
<td>KCl</td>
<td></td>
<td>nM</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>26.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>30.3 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>34.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>33.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4 represents the equilibrium dissociation constants (K_d) of Klenow-DNA binding at 500mM KCl, pH 7.9 with varying concentration of PEG 6000 Da or Ficoll 70 kDa. The osmolarity and the K_d obtained is listed for each individual solution condition and the thermodynamically linked water released (Δn_{H2O}) is listed for each series. Two series are listed for PEG 6000 Da. The data listed in Table 4 represent a single representative series of PEG 6000 Da and Ficoll 70 kDa. The values listed in the body of the text and in Figure 9 represent average value of Δn_{H2O} which incorporate the data listed in this table.

Table 9: Hydration change associated with the DNA binding by Klenow to the 13/20mer construct. PEG 6000 Da and Ficoll 70kDa were used. The experiments were performed in 500mM. The apparent numbers of water released are listed. The series listed in this table were not listed in Table 4. The average thermodynamically linked water release for each solute with multiple series is plotted and listed in Figure 9.

<table>
<thead>
<tr>
<th>Solute type</th>
<th>[Solute]</th>
<th>Π Osm</th>
<th>K_d nM</th>
<th>Δn_{H2O}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 6000 Da</td>
<td>0 0.91</td>
<td>537.3 ± 36</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 1.01</td>
<td>286 ± 23</td>
<td></td>
<td>465.7 ± 18.1</td>
</tr>
<tr>
<td></td>
<td>10 1.14</td>
<td>81.1 ± 4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 1.3</td>
<td>19.1 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 1.51</td>
<td>4.5 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ficoll 70 kDa</td>
<td>0 0.92</td>
<td>286.3 ± 29</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 1.02</td>
<td>126.6 ± 8.4</td>
<td></td>
<td>491.2 ± 14.7</td>
</tr>
<tr>
<td></td>
<td>10 1.16</td>
<td>33.8 ± 3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 1.33</td>
<td>8.2 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 1.13</td>
<td>45.0 ± 6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 1.25</td>
<td>23.4 ± 1.5</td>
<td></td>
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</table>
Figure 19: Plot of the extended non linear thermodynamic salt linkage in Kglutamate and the linear salt linkage in KCl for Klenow (panel A) and Klentaq (panel B)
Figure 19 shows a comparative plot of the non linear thermodynamic salt linkage in Kglutamate versus the linear thermodynamic salt linkage in KCl for Klenow and Klentaq. This plot provides the evidence that non linear analysis restores the linked ion release in Kglutamate to that in KCl (linearly obtained). The curves are parallel are low salt. However, an interesting observation is that in Klentaq, the linear salt linkage in KCl and the non-linear salt linkage in Kglutamate do not overlap at low salt. In the case of Klenow, the two linkages overlap at low salt. The non-linear analysis predicts that, at low salt, the osmotic component is insignificant and that if the linked ion released ($\Delta n_{H_2O}$) is similar in the two salts, then the linear salt linkage in KCl and the non linear salt linkage should overlap. The absence of overlap suggests that the presence of an anion type dependent, salt concentration independent process or event that contributes the observed energetic difference at low salt. The nature of the process is unknown and to be determined.
VITA

Daniel Deredge was born in Montelimar, France, in January 1982, to Brigitte Deredge and Dereje Wolde-Medhin. Daniel grew up for most part in Addis-Abeba, Ethiopia, where he attended the Lycee Guebre Mariam. He joined Lousiana State University in the spring 2000 to pursue an education. He obtained a bachelor of sciences degree from Lousiana State University in 2003 and entered the graduate program in the department of Biological Sciences as a doctoral student. As a graduate student, Daniel worked in the laboratory of Dr Vince J. LiCata as well taught multiple classes as a teaching assistant. After graduation in December 2009, Daniel plans to pursue a scientific career as a postdoctoral researcher in an academic institution.