Cloning, Purification, and Preliminary DNA-binding and Unfolding Results for the DNA Polymerase I from the Psychrophile Psychromonas ingrahamii

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CLONING, PURIFICATION, AND PRELIMINARY DNA-BINDING AND UNFOLDING RESULTS FOR THE DNA POLYMERASE I FROM THE PSYCHROPHILE *PSYCHROMONAS INGRAHAMII*

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Department of Biological Sciences

by

John Tod Baker

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<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
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<tr>
<td>Potassium Phosphate</td>
<td>potassium phosphate buffer made by mixing mono- and dibasic-forms of potassium phosphate</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>13/20 mer DNA</td>
<td>primed-template DNA used for all DNA-binding experiments</td>
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<tr>
<td>$K_d$</td>
<td>dissociation constant of binding</td>
</tr>
<tr>
<td>$\Delta H$</td>
<td>enthalpy change of reaction</td>
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<td>$\Delta S$</td>
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ABSTRACT

*Psychromonas ingrahamii* is a psychrophilic bacterium that lives in Arctic polar sea ice and grows at a temperature range of -12 to 10º C. This bacterium resides within veins inside the ice where the salinity is high, resulting in a freezing point depression and liquid water. The large fragment of DNA polymerase I from *Psychromonas ingrahamii*, called Klenpin, has been cloned, expressed, and purified in our laboratory. Although enzyme kinetic studies have been performed on a few psychrophilic enzymes, the thermodynamics of ligand binding and of protein stability have not been well studied for this class of extremophilic proteins. Here we present initial results on the salt dependence and temperature dependence of DNA-binding for Klenpin as well as unfolding results obtained for this protein. The KCl dependence shows that DNA-binding affinity decreases as the salt concentration is increased from 10 mM to 1 M. Salt addition titrations with KCl show that salt displaces the polymerase from the DNA up to approximately 0.5M KCl, after which increased [KCl] induces rebinding of the polymerase to the DNA. Rebinding of the polymerase to DNA continues to increase up to 4.3 M KCl. Such rebinding to DNA at high salt has previously been documented in glutamate salts for several polymerases, but rebinding in KCl has not been previously observed for any polymerase. Temperature dependence shows Klenpin binds to DNA with submicromolar affinity at 8º C but binding affinity increases as the temperature is raised to 25º C. Unfolding results show that under similar solution conditions Klenpin has a similar $T_m$ of 62.7ºC to its mesophilic homolog from *E. coli*, Klenow. This $T_m$ is slightly higher than that for Klenow.
CHAPTER 1: INTRODUCTION

DNA Polymerase I is one of the enzymes that participates in the replication and repair of DNA in prokaryotes. This polymerase consists of one subunit and has three enzymatic activities: (1) DNA polymerization (2) 3’-5’ exonuclease activity and (3) 5’-3’ exonuclease activity. The 3’-5’ exonuclease activity functions in the proofreading of DNA. The 5’-3’ exonuclease activity makes the DNA Polymerase I from *Escherichia coli* capable of performing a coordinated polymerization and 5’-3’ exonucleolytic degradation known as nick translation and appears to be essential for cell viability in *Streptococcus pneumoniae* (1, 2, 3, 4). Removal of the 5’-3’ exonuclease domain produces what is called the “large fragment,” which has its DNA polymerization and 3’-5’ domains left intact (1). The large fragments from the enzymes of *Escherichia coli*, *Thermus aquaticus*, and *Deinococcus radiodurans* have been previously characterized thermodynamically (10, 12, 13, 20, 21, 22). The large fragment from the psychrophile *Psychromonas ingrahamii*, which is the subject of this thesis, has never been cloned, purified, or thermodynamically characterized. This large fragment will be called Klenpin in analogy to the names Klenow, Klentaq, and Klendra, the large fragments from *Escherichia coli*, *Thermus aquaticus*, and *Deinococcus radiodurans*. The thermodynamic binding profiles for DNA polymerases studied thus far suggest little to no DNA-binding will occur at subzero temperatures and thus a thermodynamic profile explaining the DNA synthesis observed in psychrophiles (23, 24) has yet to be discovered. This thesis will present a cloning and purification methodology as well as some preliminary unfolding and DNA-binding data for Klenpin.

*Psychromonas ingrahamii* is a psychrophilic bacterium that lives in Arctic polar sea ice and grows in a temperature range of -12 to 10°C. The organism was isolated from sea ice near Point Barrow, Alaska. -12°C is the lowest growth temperature of any organism authenticated by
a growth curve (Figures 1 and 2) (5, 6). This bacterium resides in veins within the ice where the salinity is high and freezing point depression maintains the water in a liquid state. The salinity in the brine pockets can be as high as 150 ‰ (150 g salt/kg water ≈ 2.6 M) (5).

Figure 1: Arctic polar sea ice located in Point Barrow, Alaska, which is the area Psychromonas ingrahamii was isolated (Photo source: 7).

Figure 2: Growth curve of Psychromonas ingrahamii at -12°C. Values represent averages of 16 optical density readings (data and figure are from reference 5).
Psychrophiles thrive in permanently cold environments including those which are at subzero temperatures. Some of the challenges that low temperature presents to organisms are the reduction of the rate of enzyme-catalyzed reactions, the decrease of molecular motions associated with protein function, the reduction in rate of proline isomerization for folding, and increased viscosity of water. There have been a number of kinetic studies performed on psychrophilic enzymes, which have revealed some common properties such as high specific activity, heat inactivation due to increased flexibility rather than unfolding, and weak substrate binding (8). However, the thermodynamics of substrate binding and stability of psychrophilic enzymes have not been well characterized. The thermal unfolding and DNA-binding data for Klenpin presented in Chapter 4 accomplishes a number of goals of a preliminary thermodynamic characterization: (1) a measurement of the Tm under the solution conditions used for binding experiments (2) a comparison of binding affinity at an acidic and basic pH (3) a salt dependence of binding that probes salinities occurring in the brine pockets of Arctic sea ice (4) a temperature dependence of binding affinity in the range 8-25⁰C and (5) application of thermodynamic analyses to the temperature and salt dependencies.

The Gibbs-Helmholtz equation can be used to analyze the effect of changing the temperature on the association constant of substrate binding. Figure 3 shows examples of Gibbs-Helmholtz plots of ΔG versus temperature. Enthalpy and entropy can be determined from such plots as a function of temperature as well as any changes in heat capacity associated with binding. A large negative heat capacity change (ΔC_p) is commonly observed for DNA-binding proteins, especially DNA sequence dependent binding proteins (9, 10). The shape of the Gibbs-Helmholtz curve when there is a negative ΔC_p is concave up as shown in Figure 3C. A correlation between the magnitude of the negative ΔC_p and the burial of hydrophobic surface
area has been commonly observed (9). For DNA-binding proteins studied thus far, the range of
temperatures examined experimentally has not included subzero temperatures that occur in
Arctic sea ice. Extrapolation of existing experimental data on other polymerases to subzero
temperatures results in very low association constants of binding especially at temperatures as
low as -12 degrees (10, 25, 11). However, both psychrophilic prokaryotic and eukaryotic
microorganisms have been shown to carry out DNA and protein synthesis at subzero
temperatures (23, 24). In fact, the growth curve for Psychromonas ingrahamii is an example of
such synthesis. Thus, a thermodynamic profile for DNA binding in psychrophiles has not been
discovered. The DNA binding affinities sufficient for DNA synthesis at subzero temperatures
can be thermodynamically accomplished by either shifting a Gibbs-Helmholtz curve (e.g. Figure
3B) to the left or via an inversion of the concave up Gibbs-Helmholtz curves observed for DNA
polymerases thus far. Inversion of the Gibbs-Helmholtz curve to concave down would change
the $\Delta C_p$ of binding to a positive value and would be the first time burial of charged surface
outweighed the burial of hydrophobic surface for a DNA binding protein.

Salt effects can be characterized thermodynamically by measuring the association
constant of binding or % saturation at different salt concentrations. For DNA-binding proteins, a
typical result is the inhibition of binding as the salt concentration is raised (Figure 4). To date,
the only exception to this includes a couple of homologous systems to Klenpin in high
concentrations of potassium glutamate (13) and one system from a hyperthermophilic Archaeon
in KCl (14). The variation of the association constant with salt concentration can be linked to an
associated release or uptake of ions from the reacting macromolecules upon binding. The
thermodynamic effects of KCl on Klenpin DNA binding at salt concentrations probing the
salinities of the brine pockets was observed to promote DNA binding. The inhibitory effect of
KCl observed for all other DNA polymerases and virtually all DNA binding proteins (the only one exception mentioned above from reference 14) means that increasing the KCl concentration would not be a mechanism for shifting DNA-binding protein’s Gibbs-Helmholtz curves leftward into subzero temperatures. However, the enhancement of binding at high KCl concentration for Klenpin does suggest this as a possible mechanism for shifting the polymerase’s Gibbs-Helmholtz curve leftward into sufficient affinities for DNA synthesis at subzero temperatures.

Before presenting the thermal unfolding and DNA-binding data for Klenpin, two chapters on the cloning and purification of Klenpin will be discussed.

Figure 3: Examples of Gibb-Helmholtz plots (9) for reactions without (A) and with (B) a $\Delta C_p$. $\Delta H$ and $\Delta S$ do not vary with temperature in panel A which results in the plot being linear.
Figure 4: Single site binding isotherms at 25°C with the Klenpin homolog Klenow (12). The binding affinity decreases as the KCl concentration is raised. The KCl concentrations were 250 mM (diamonds), 300 mM (upward triangles), 400 mM (downward triangles), 450 mM (squares), 500 mM (circles).
CHAPTER 2: CLONING OF KLENPIN GENE

The goal of the cloning was to overexpress a D434A mutant Klenpin in an *E. coli* expression strain. The purpose of the D434A mutation is to delete the 3’-5’ exonuclease activity analogous to the D424A exo' mutant of Klenow polymerase which has been used in the majority of studies on that polymerase (15). Binding studies require this deletion because without it the exonuclease activity degrades the DNA substrate. As will be mentioned in Chapter 4, the deletion appears to be effective. The cloning methodology used is as follows.

Colonies of *Psychromonas ingrahamii* were generously provided by Dr. Brent Christner at LSU. To obtain genomic DNA, the first step was to grow 1 mL cultures of *P. ingrahamii* in Marine Broth (VWR Inc.) at its optimum growth temperature 5°C. Marine Broth is used to cultivate heterotrophic marine bacteria. Yeast and peptone extract provide nitrogen, vitamins, and minerals and high salt content helps simulate sea water. Numerous minerals are also included to duplicate the major mineral composition of sea water. The 1 mL cultures were grown for 8-12 days until they reached an O.D.600 of about 0.9. Cultures were spun down in 1000 µL aliquots in 1.5 mL Eppendorf tubes and genomic DNA was accessed from the cell lysates as PCR can be done within such a cell lysate. A pellet was resuspended in 100 µL of dH$_2$O, heated to 90°C for 15 minutes in a water bath, and spun down again. The cell lysate supernatant contained the genomic DNA. PCR was performed on the cell lysate using a forward primer that contained an Nde I restriction site and a reverse primer that contained a Xho I restriction site and a stop codon (Figure 5). The primers flanked the ends of the Klenpin gene (the full length polymerase and the Klenpin large fragment amino acid sequence are shown in Figure 6) and the PCR produced a linear DNA product that was subsequently double digested with Nde I/Xho I restriction enzymes. This fragment was then ligated into a Nde I/Xho I double digested pET 24a vector (from Novagen). The resulting plasmid was transformed into an
expression strain, and overexpression can be induced by the addition of IPTG.

HMS174(DE3)pLysS competent cells were used as the expression strain during purification.

This plasmid was also used to create the D434A mutant which is also expressed from the pET24a vector. The aspartate that was mutated is highlighted in Figure 6. We collaborated with Dr. Fred Moshiri at Monsanto for the cloning and mutagenesis of the Klenpin gene.

**Forward Primer:**

```
GCTGACCATATGGGGCACAGCAGCATTT
GATCGCAGCGGCTACAAAAC
```

**Reverse Primer:**

```
GTCAGCCTCGAGTTAATGCGCCTC
GTCCCAATTATCACC
```

Figure 5: Forward and reverse primers used for the PCR of the *P. ingrahamii* genomic DNA. The number of nucleotides complementary to the Klenpin sequence for these primers satisfies the primer design guideline of having 18-21 basepairs of complementary sequence. The Nde I restriction site for the forward primer and the Xho I restriction site for the reverse primer are underlined.
Figure 6: Psychromonas ingrahamii full length DNA Polymerase I amino acid sequence from the sequenced genome in PubMed. The first amino acid of Klenpin (S) is in bold and underlined and an added methionine and glycine precede the highlighted serine. These added amino acids are not part of the natural sequence and were added because an N-terminal MGSS has been shown to increase the expression level of some proteins. The aspartate to be mutated to an alanine is also in bold and underlined (D). The protein id number for the full length polymerase in PubMed is YP_944472. Full Length: 938 aa. Klenpin: 613 aa
CHAPTER 3: KLENPIN PURIFICATION.

HMS174(DE3)pLysS competent cells were used as the expression strain. These cells were transformed with the pET24a/D434A-Klenpin plasmid and then plated on LB-agar plates. LB minicultures (5 mL) were created with the colonies from the plates and were grown for 16-18 hrs at 37°C. The minicultures were then used to inoculate 1 L LB cultures (4, 1 L cultures total, each culture was inoculated by pouring in 2 minicultures) which were grown at 37°C until they reached an O.D.600 of 0.6. Then, the cultures were cooled outside the shaker-incubator for 30 minutes followed by the addition of 1 mM IPTG followed by induction inside the shaker-incubator for 24 hrs at room temperature. During the 30 minute cooling period, the shaker-incubator was left open and the internal temperature would cool to about 27-29°C before the liter cultures were put back inside (temperature control of shaker-incubator was subsequently turned off for the 24 hour room temperature growth). After the 24 hour induction, the cells were harvested at 7000 RPM (8671 RCF (xg)) for 20 minutes, at 4°C. The pellets were then resuspended in 4X weight (in g) of lysis buffer (50 mM Tris-HCl, 2 mM EDTA K+ form, 1 mM DTT, pH 7.5). Then 1 mM PMSF and 4 mg/mL lysozyme were added, incubated for 15 minutes (on ice) and then centrifuged at 10,000 RPM (12096 RCF (xg)) for 30 minutes, at 4°C. The supernatant was treated with the basic polymer PEI (polyethyleneimine) for the next steps. PEI precipitation is commonly used in strategies for the purification of DNA-binding proteins (17). PEI is positively charged at neutral pH and precipitates DNA and acidic proteins at low ionic strength by forming charge neutralization complexes and cross bridges between the complexes.

The PEI precipitation strategy that was used for the purification of Klenpin aimed to precipitate Klenpin and DNA away from proteins and then to elute Klenpin from the pellet by resuspending the pellet in high salt buffer. The procedure was as follows. The supernatant was decanted from the lysate pellet (on ice) and 0.2 % PEI was added using a 10% PEI/dH2O, pH 7 stock. Then 0.1
% PEI was added until no more precipitate formation was seen. The resulting suspension was incubated for 15 minutes and then centrifuged at 10,000 RPM (12096 RCF (xg)) for 30 minutes, at 4°C. The supernatant was discarded and the pellet was washed in a higher salt PEI wash buffer (10 mM KH₂PO₄, 2 mM EDTA Na⁺ form, 1 mM DTT, 150 mM NaCl, pH 7) using a homogenizer. The suspension was then centrifuged at 10,000 RPM (12096 RCF (xg)) for 40 minutes, at 4°C. The supernatant was then brought to 42% (NH₄)SO₄ based on calculations made by the EnCor Ammonium Sulfate calculator set at 25°C (18). After a 30 minute incubation at 4°C, the suspension was centrifuged at 10,000 RPM (12096 RCF (xg)) for 4 minutes, at 4°C. The supernatant was taken and brought to 60% (NH₄)SO₄ using the EnCor calculator (18). After 24 hours, the 60% (NH₄)SO₄ suspension was centrifuged at 10,000 RPM (12096 RCF (xg)) for 40 minutes, at 4°C. The pellet contained Klenpin and was redissolved in 10 mM potassium phosphate, pH 7.5. This solution was loaded onto a BioRex-70 (Bio-Rad, Hercules, CA) cation exchange purification column that was equilibrated to the same buffer and a wash was performed with several column volumes of the buffer. The purpose of the BioRex column was to bind PEI and some E. coli proteins to the column while allowing Klenpin to flow through. Fractions containing protein were identified using a spectrophotometer at 280 nm. The fractions containing protein were concentrated to 1 mg/mL or above by assuming the absorbance was primarily due to Klenpin (an extinction coefficient for Klenpin was calculated using the ExPASy ProtParam tool, ε=52260 M⁻¹ cm⁻¹) (19). The concentrate was dialyzed against 1 L of 10 mM Potassium Phosphate, pH 7.0 for 1 hour. This dialysis was repeated three more times with 1 L of fresh buffer. The dialyzed protein was loaded onto a Type I, 80 µM ceramic hydroxyapatite (Bio-Rad, Hercules, CA) column and washed with 5-10 column volumes of the same buffer. The wash was followed by a 10 mM-225 mM potassium phosphate gradient. The
absorbance of the fractions at 280 nm after the addition of the gradient was measured to identify which ones contained protein. The fractions containing protein that followed the two highest absorbance fractions were collected and dialyzed into storage buffer (10 mM Tris Base, 0.5 mM DTT, 50 mM KGl, 50% Glycerol, pH to 7.5, pH’d with either glutamic acid or HCl). The protein was dialyzed against 2 L of storage buffer for 2 hrs. and then this was repeated with 2 L of fresh buffer. The appearance of purified Klenpin on a SDS-PAGE gel is shown in Figure 7.

Figure 7: Appearance of purified Klenpin on a SDS-PAGE gel. Lane 2= Ladder, Lanes 3-6=dilutions of purified Klenpin. The band corresponding to Klenpin is the highest intensity band for lanes 2-6.
CHAPTER 4: PRELIMINARY DNA-BINDING AND UNFOLDING RESULTS

Methods

Thermal denaturation of Klenpin was monitored by circular dichroism at 222 nm using a Jasco J-815 Model CD Spectrometer. A Klenpin concentration greater than 1 mg/ml was used. Klenpin was incubated somewhere between 2-10 minutes at each temperature before measuring the CD signal. Klenpin was in KTA buffer, with a pH of 7.9 at 25°C (the change in pH with increasing temperature was not recorded), which contained 20 mM Tris-HCl, 0.1 mM EDTA, 22 mM ammonium sulfate, 1 mM DTT, 10% glycerol, and 1 mM PMSF.

Fluorescence anisotropy was employed to measure binding affinity under various solution conditions. For this technique, a fluorophore is attached to DNA. For binding experiments with Klenpin, Rhodamine-X (ROX) was attached. When protein binds to the DNA, the rotational diffusion of the fluorophore decreases and the anisotropy signal increases. By performing a protein-DNA titration, the increase in anisotropy signal creates a binding curve from which a dissociation constant of protein-DNA binding can be obtained ($K_d$) (Figure 8). The $K_d$ can be related to the free energy change of binding by the equation:

$$\Delta G = -RT\ln K_d$$ (Equation 1)

The anisotropy data collected for Klenpin titrations was fit to a single-site binding isotherm using the equation:

$$\Delta A = \left\{\frac{\Delta A_T(E_T/K_d)}{(1+E_T/K_d)}\right\}$$ (Equation 2)

where $\Delta A$ = the change in anisotropy at each point, $\Delta A_T$ = total change in anisotropy, $E_T$ = enzyme concentration at each point, and the $K_d$ = dissociation constant.

The DNA used for all experiments was 13/20 mer at a concentration of 1 nM (Figure 9). Complete lists of buffer components for experiments are provided in the captions of the figures.
Figure 8: Examples of Protein-DNA titrations under different solution conditions using the Fluorescence anisotropy binding assay (Data and figure from reference 13).

13/20 mer

ROX-5’–TCGCAGCCGTCGA- 3’

3’-AGCGTCGGCAGGTTCCCCAAA-5’

Figure 9: Sequence and ROX label location for the 13/20 mer DNA substrate.

Thermal Unfolding

Thermal denaturation of Klenpin monitored by circular dichroism is shown in Figure 10. The melting temperature for Klenpin is 62.7°C. The highest temperature used during purification of Klenpin is 25°C-30°C and the highest temperature used for binding experiments with Klenpin is 25°C. Interestingly, this T_m for Klenpin is about seven degrees higher than the T_m for Klenow, its mesophilic homolog from E. coli under the same solution conditions (10). This result differs from other unfolding studies on psychrophilic/mesophilic/thermophilic homologous enzyme sets.
in which the melting temperature increases or decreases with the physiological temperature of the organism (8). It is important to note again that Klenpin is the large fragment of the \textit{P. ingrahamii} full length polymerase and lacks the 5’-3’ exonuclease domain. The \(T_m\) for Klenpin determined from Figure 10 may be different than the \(T_m\) for the full length polymerase. The direction of change in \(T_m\) upon addition of the 5’-3’ exonuclease domain to Klenpin would be a topic of interest in light of the comparative thermal denaturation data obtained for the Klenpin homologs Klenow and Klentaq with their full length counterparts (27) where the influence of adding the 5’-3’ exonuclease domain on the stability of the rest of the protein was opposite for the two proteins. Addition of the 5’-3’ exonuclease domain stabilized Klenow but destabilized Klentaq (27).

Figure 10: Thermal denaturation of Klenpin monitored by circular dichroism. Klenpin was incubated at each temperature somewhere between 2-10 minutes before measuring the CD signal. Klenpin was in KTA buffer, at pH 7.9, which contained 20 mM Tris-HCl, 0.1 mM EDTA, 22 mM ammonium sulfate, 1 mM DTT, 10% glycerol, and 1 mM PMSF.

**DNA-Binding Solution Conditions**

For DNA-binding experiments, two solution variables had to be optimized. One was a pH at which binding affinity is great enough for experiments not to require excessive amounts of
protein. The other condition is related to the presence of exonuclease activity in the purified protein from some, but not all purifications. No exonuclease activity was observed for the purified Klenpin used for the titrations in Figure 13, but the inconsistency in exonuclease activity from purification to purification requires that the purified protein must be screened for exonuclease activity in each purification. This exonuclease activity degrades the DNA that is used for the binding assay (See Figure 11). The exonuclease activity is likely due to contaminating exonucleases from the E. coli expression strain rather than the D434A mutation not being effective at deleting the 3’→5’ exonuclease activity of Klenpin. This is because no exonuclease activity was observed with Klenpin from purifications later on in the project under identical solution conditions as in Figure 11 (Compare Figure 11 to Figure 12). Most binding experiments in this thesis were done under solution conditions where no exonuclease activity was observed over periods of time much longer than a fluorescence anisotropy titration.

Figure 11: Loss of anisotropy signal that occurs when 13/20 mer DNA is titrated with Klenpin in a buffer containing 10 mM KCl, 2 mM PIPES, pH 6, 25°C. The loss of signal likely occurs due to exonuclease activity present in purified Klenpin. No loss of signal was seen when Klenpin from purifications later on in the project was used under identical solution conditions (Compare to Figure 12).
Figure 13 shows titrations with initially purified Klenpin performed at two different pH’s and a temperature of 25°C. 25°C was the temperature used for a number of experiments with Klenpin because the salt dependencies for Klenpin’s homologs were done at 25°C. The binding affinity at pH 6 is at least 4X higher than at pH 7.9. Also, at pH 7.9 binding appears to be unusually weakly dependent on salt concentration as binding at 50 mM KCl showed almost the same Kd value as binding at 500 mM KCl.
One approach to reducing exonuclease activity is to add the divalent-cation chelater EDTA because many *E. coli* exonucleases and DNA polymerases require Mg\(^{2+}\) as a cofactor for their exonuclease activity. Figure 14 shows a titration performed at an EDTA concentration of 10 mM. The fact that the anisotropy signal never decreases during the entire titration (about 4 hours) supports that the exonuclease activity has been reduced substantially. More importantly, the anisotropy signal was checked 24 hours later and it was the same as the signal shown on the plateau of the titration curve. However, the addition of 10 mM EDTA reduces binding affinity because it removes the Mg\(^{2+}\) cofactor from Klenow polymerase. Although the titration in Figure
that was done at pH 6 in the absence of EDTA has a higher $K_d$ than the EDTA titration in Figure 14, the difference in binding affinity is due to a difference in KCl concentration (10 mM vs. 50 mM).

Figure 14: A titration performed at an EDTA concentration of 10 mM to reduce exonuclease activity. The titration was done at 25°C and the other buffer components are listed above the graph along with the $K_d$ for binding to the 13/20 mer DNA. The fact that the anisotropy signal never decreases during the entire titration (about 4 hours) supports that the exonuclease activity has been reduced substantially. The anisotropy signal was also checked 24 hours later and it was the same as the signal shown on the plateau of the titration curve.
Salt Dependence of DNA-binding

A set of 4 titrations were performed at different concentrations of KCl below the salinity of the Arctic sea ice brine pockets and the data were fit to a single-site binding isotherm. Figure 15 shows the fitted data and the associated estimates of the $K_d$ for these titrations. The $K_d$s are considered estimates because the titrations consumed an unexpected amount of protein and the plateau was not reached for 3 of the titrations. In this range of KCl concentrations, the affinity decreases as the concentration is raised up to 1 M.

![KLP Salt Dependence](image-url)

Figure 15: Titrations performed at different concentrations of KCl. The data were fit to a single-site binding isotherm and estimated dissociation constants ($K_d$) were obtained. The dissociation constants reported in the figure are in units of nM.
The variation of the association constant with salt concentration can be linked to an associated release or uptake of ions from the reacting macromolecules upon binding ($\Delta N_{\text{ions}}$) by making a Wyman Plot. The $\Delta N_{\text{ions}}$ is equal to the slope of a $\ln(1/K_d)$ vs $\ln[\text{KCl}]$ plot (See Equation 3). The thermodynamically linked participation of ions was an ion release of $\Delta N_{\text{ions}} = -0.8$ in Figure 16. Ion releases in KCl (negative $\Delta N_{\text{ions}}$) were reported for the Klenpin homologs from \textit{E. coli}, \textit{T. aquaticus}, and \textit{D. radiodurans} but were larger having a $\Delta N_{\text{ions}}$ of -4.9, -3.4, and -5.2 respectively. The Wyman Plots for the mesophilic homolog from \textit{E. coli} and the thermophilic homolog from \textit{T. aquaticus} are shown in Figure 17 (12, 13, 22).

$$\frac{\{dln(1/K_d)\}}{\{dln[\text{KCl}]\}} = \Delta N_{\text{ions}} = \Delta n\text{Cations} + \Delta n\text{Anions} \quad \text{(Equation 3)}$$

![Figure 16](image-url)
In order to conserve protein, concentrations higher than 1 M [Salt] were probed using a salt addition experiment. For this experiment, the protein and DNA are loaded at saturation and the salt concentration is increased by adding high salt concentration titrant. The normalized anisotropy signal is monitored to determine the % saturation at a given salt concentration (Figure 18). The displacement of Klenpin from the DNA up until 1 M KCl is consistent with the dependence shown in Figure 15. However, when the concentration is increased above 1 M, a re-association of Klenpin with the DNA is observed. This re-association continues up to the highest KCl concentration used (4.3 M) such that the % saturation is 67 %. This range of data probes the salinities that occur in Arctic sea ice brine pockets (a salinity of 150 ‰ ≈ 2.6 M salt). The displacement and subsequent re-association of Klenpin with the DNA is the first observation of such a KCl dependence for DNA-binding proteins, however a displacement followed by a re-association at higher salt concentrations has been observed for a couple of homologous large
fragments in the salt KGl u (13). The salt addition experiments for these other homologs in KCl and KGl u are shown in Figure 19. For the Klenow and Klentaq experiments with KCl, the typical inhibition of binding affinity with increased salt concentration is observed over the entire range of KCl concentrations but a re-association occurs with the DNA at high concentrations of KGl u. It is of relevance to note that an increase in binding affinity with increasing KCl concentration was the only dependence observed for experiments done in the KCl concentration range 1-1.6 M with the TATA-Binding Protein from the hyperthermophile *Pyrococcus woesei*. *P. woesei* lives in the highly saline conditions near found near deep sea vents (14).

![Figure 18](image_url)

*Figure 18: Salt addition experiment with KCl. Klenpin and DNA are loaded at saturation and the anisotropy signal is measure as high [KCl] is added. The normalized anisotropy is equal to the %saturation. The concentration of KCl increases up to 4.3 M by the end of the experiment.*
Temperature Dependence of DNA-binding

Binding affinity was measured at three different temperatures: 8, 16, and 25°C (Table 1). Figure 20a shows this dependence with an associated best-fit line. An authentic linear temperature dependence corresponds to a change in heat capacity of the reaction that is equal to zero. However, the somewhat limited range of temperatures used is not large enough to rule out
potential curvature that would result from a non-zero $\Delta C_p$. If it is assumed that the dependence is linear, then a $\Delta H$ of binding is constant with temperature. The data in Table 1 can be used to make a Van’t Hoff plot (not shown) and the $\Delta H$ can be calculated. This calculation for Klenpin revealed a $\Delta H$ of -7.9 kcal/mol. The temperature effect on DNA-binding for Klenpin and its mesophilic homolog from *E. coli* (Klenow) can be compared using the linear portion of the Gibbs-Helmholtz curve for Klenow shown in Figure 20b. The slope of the dependence for Klenpin is about 1.25X larger than the slope for Klenow. The temperature dependence for Klenpin was done in a lower [KCl] buffer than for Klenow but the KCl dependencies for the polymerases can be compared because their salt dependencies are linear at the KCl concentrations of the buffers used (Figure 16, 17, 18). The curvature seen for Klenow in Figure 20b indicates a non-zero change in heat capacity. In this case, the concave up curve means the change in heat capacity is negative. The Gibbs-Helmholtz curve for Klenpin’s thermophilic homolog Klentaq is shown in Figure 20c and also has a concave-up curvature which indicates a negative change in heat capacity. Gibbs-Helmholtz analysis of these curves with a non-zero heat capacity change results in the $\Delta H$ varying with temperature (See reference 10 and 25) (a constant $\Delta H$ was assumed for Klenpin above). The data for Klenow and Klentaq in the 5-25°C linear range were used to make Van’t Hoff plots as was done above for Klenpin and similar $\Delta H$s were calculated of -7.2 kcal/mol for Klenow and -8.7 kcal/mol for Klentaq (10, 25).

For the Klenpin temperature dependence shown in Figure 20a, the free energy of binding becomes more positive (lower affinity) as the temperature is lowered towards subzero temperatures. Hypothetically if this relationship were to reverse at lower temperatures, there would be a resulting concave down curve and positive change in heat capacity which differs from the large negative heat capacity change for other DNA-binding proteins (10). A large
negative heat capacity change is commonly correlated with the burial of hydrophobic surface area upon binding, thus a significantly different binding interface would required if further studies were to find such a reversal of Gibbs-Helmholtz curvature.

Table 1: Binding affinity for Klenpin-13/20 mer DNA binding measured at 3 different temperatures and in a buffer containing 10 mM KCl, 2 mM PIPES, 10 mM EDTA, pH 6.0.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>K_d (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>581.12</td>
</tr>
<tr>
<td>16</td>
<td>404.22</td>
</tr>
<tr>
<td>25</td>
<td>259.36</td>
</tr>
</tbody>
</table>

Figure 20: (a) Temperature dependence of Klenpin-DNA binding. The linear equation is shown in as well. (b) Temperature dependence of DNA-binding for Klenpin’s mesophilic homolog Klenow (10). (c) Temperature dependence for Klenpin’s thermophilic homolog Klentaq (boxes) and its full-length counterpart Taq (circles) (25). (Figure Con’t).
Possible Stoichiometric Issue

For continuation of the study of Klenpin DNA-binding it is important to be aware of a possible 2:1 stoichiometry of Klenpin 13/20 mer binding. In order to properly use a single-site binding isotherm to determine dissociation constants, the stoichiometry of binding must be 1:1. Figure 21 shows an initial stoichiometric titration with Klenpin using 13/20 mer DNA. The stoichiometry determined from this curve is 1.78. This raises the question of whether binding is
to 13/20 mer is 2:1. Binding of the Klenow, Klentaq, and Klendra polymerases to this 13/20 mer DNA show a 1:1 stoichiometry (12, 22). It is possible that the stoichiometry is showing up this way because the binding is low affinity for Klenpin. If increasing binding affinity through changing solution conditions (such as a lower concentration of EDTA) is not an option, using Isothermal Titration Calorimetry (ITC) may be a way to observe 1:1 stoichiometry. Also, if low affinity binding is causing the stoichiometry to show up 2:1, work has been done on ITC to analyze low affinity binding data that give a false 2:1 stoichiometry. It is also possible to use another DNA for which the measured stoichiometry is 1:1. A primed template DNA was designed that is similar to 13/20 mer with a hairpin loop on one end and is shown in Figure 22. The loop is supposed to prevent the polymerase from binding to that end. Future binding experiments should compare this oligonucleotide to the 13/20 mer used for the studies in this thesis.

Figure 21: Stoichiometric titration for Klenpin binding to the 13/20 mer DNA used for all experiments. The stoichiometry and a fitting parameter key are shown above the graph.
Figure 22: Hairpin DNA hp28 with 13/20 overhang and adenine to thymine substitution at position 22 for ROX labeling. This DNA was designed to prevent 2:1 polymerase binding. The structure was generated by the IDT Oligoanalyzer (Integrated DNA Technologies, San Jose, CA). Its sequence is shown below with the thymine substitution shown in red:
5’-AAACCCTGATCGGACGTCGGA\textcolor{red}{T}ACGACGTC\textcolor{red}{G}ATC-3’
CHAPTER 5: DISCUSSION

The gene for the large fragment DNA Polymerase I from the psychrophilic bacterium *Psychromonas ingrahamii* was successfully cloned into a pET24a expression vector that allows for IPTG induction. The Klenpin used for experiments is a D434A mutant created by mutagenesis. It appears that this mutation was effective at substantially reducing the 3’-5’ exonuclease activity of Klenpin involved in proofreading. A purification strategy was developed that yields active Klenpin consistently from purification to purification. As will be discussed below, the highest temperature utilized during the purification of Klenpin is much less than the measured $T_m$ for the protein. The purity of Klenpin on the gel in Figure 7 is comparable to other polymerases that have been purified (26).

Thermal denaturation of Klenpin monitored by circular dichroism yields a $T_m$ of 62.7ºC. The highest temperature used during purification of Klenpin is 25ºC-30ºC and the highest temperature used for binding experiments with Klenpin is 25ºC. This indicates the absence of any unfolded Klenpin present during titrations. Furthermore, raising the overexpression temperature for the Klenpin purification could lead to a higher yield of Klenpin. Considering the quite weak µM binding affinity observed above 40 mM KCl, raising this temperature could be a worthwhile way to increase the yield of protein and hence the number of experiments possible from each purification. In comparison to the homologous large fragment from the mesophile, *E. coli*, the $T_m$ for Klenpin is about seven degrees higher (10). This result differs from other unfolding studies on psychrophilic/mesophilic/thermophilic homologous enzyme sets in which the melting temperature increases or decreases with the physiological temperature of the organism (8). This raises the possibility that Klenpin is relatively inflexible for a psychrophilic protein.
The first three binding experiments performed showed that binding affinity was tighter at acidic pHs (See Figure 13 for these experiments). All other binding experiments were performed at pH 6. The presence of exonuclease activity as seen by a decrease in anisotropy signal required the addition of EDTA. 10 mM EDTA was used and this contributes to lowering binding affinity. Future work could find a possible lower EDTA concentration to be used in order to increase affinity.

A salt dependence of binding was conducted in the range 10 mM-1 M KCl. Increasing the salt concentration was seen to be inhibitory in this range. The linked participation of ions in this range was an ion release of $\Delta N_{\text{ions}} = -0.8$. This number may not represent the actual participation of ions in the binding reaction because of the preliminary nature of the titration curves and not taking into account the reversal of the KCl dependence above 1 M seen in the salt addition experiment.

The LiCata lab (13) working with Klenpin homologs observed one of the only other examples of a reversal of salt dependence for DNA-binding proteins. However, the reversal was not observed in KCl but in KGlu. In fact, the reversal was interpreted as being due to an osmotic effect (involving a lowering of the activity of water) specific to the presence of the osmolyte glutamate. However, if the KCl doesn’t lower the water activity enough to exert this osmotic effect, the question is raised as to how $K^+$ and $Cl^-$ ions might cause the reversal seen with Klenpin. $K^+$ ions are known to condense on negatively charged DNA and thus they participate as competitors to DNA-binding proteins. $K^+$ ions can also site-bind to proteins. A case of $K^+$ site-binding that is quite interesting in light of the Klenpin data is the site-binding of $K^+$ to the binding interface of the TATA-Binding Protein (TBP) from the hyperthermophile *Pyrococcus woesei* (16). *Pyrococcus woesei* lives in the highly saline conditions found near deep sea vents.
A KCl dependence for *PwTBP* was measured in the 1-1.6 M range and showed a positive relationship between binding affinity and salt over the entire range (14). This study was followed by site-directed mutagenesis of acidic residues at the binding interface which indicated uptake of K⁺ ions upon DNA-binding (16). If similar acidic residues are at the binding interface of Klenpin, this could explain the binding enhancement at high [KCl]. If acidic residues on Klenpin reached a threshold level of K⁺ saturation at concentrations above 1 M K⁺, this could provide a way to counteract the inhibitory effect of K⁺ condensation on DNA and reverse the KCl dependence.

Another interesting thing about the KCl dependence for Klenpin is that the reversal occurs at salinities present in the Arctic sea ice brine pockets (≥2.6 M). DNA and protein synthesis has indeed been observed for some psychrophilic prokaryotes and eukaryotes at low temperatures. *Psychromonas ingrahamii* and *Rhodotorula* and *Cryptococcus* yeast species have documented generation times at subzero temperatures and the cells of *Psychrobacter* species were shown to incorporate [³H]thymidine as well [³H]leucine (23, 24). Thus, unlike the effect of KCl on other DNA polymerases thus far, the enhancing effect of high concentrations of KCl on Klenpin-DNA binding could provide a mechanism of shifting the Gibbs-Helmholtz leftward into sufficient binding affinities for DNA synthesis at low temperature.

The temperature dependence of Klenpin DNA-binding is somewhat unexpected in terms of physiological correlation in a couple of ways. One is that the binding affinity decreases as the temperature is lowered towards the subzero temperatures that Klenpin lives at. The continuation of this trend would correlate with a very slow generation time for *P. ingrahamii*. This trend would also be consistent with the extrapolation of the Gibbs-Helmholtz curves for Klenpin’s homologs to subzero temperatures. The possible reversal of this trend at some temperature lower
than 8°C would better correlate with *P. ingrahamii*’s physiological growth temperatures but would be unexpected thermodynamically for DNA-binding proteins. This is because the shape of this Gibbs-Helmholtz curve would be concave down which corresponds to a positive change in heat capacity of binding. All DNA binding proteins studied thermodynamically thus far have large negative changes in heat capacity. It is possible that in Klenpin there is an exposure of hydrophobic surface area upon binding in contrast to the burial of hydrophobic surface area commonly correlated with DNA binding proteins and their negative changes in heat capacity. However, the reversal of KCl dependence at physiological concentrations discussed above provides a mechanism for which Klenpin’s Gibbs-Helmholtz curve could still be concave up and yield a negative change in heat capacity of binding like other DNA-binding proteins. The effect of KCl could shift the Gibbs-Helmholtz curve leftward to lower temperatures and sufficient binding affinities for DNA synthesis at subzero temperatures and would explain *Psychromonas ingrahamii*’s ability to grow at temperatures as low as -12°C. This would mean that Klenpin could match the thermodynamic profiles for all DNA binding proteins and include a negative change in heat capacity of binding, yet still bind with high affinity at low temperature. The effect of KCl on Klenpin shown in this thesis thus does indicate something new about the possible thermodynamic profiles of DNA-binding proteins. It indicates a potential new role for how either K⁺, Cl⁻, or both ions participate in DNA polymerase-DNA interactions. It is hoped that the work presented within this thesis will give the prospect of future work on Klenpin considerable momentum.
REFERENCES


11. Unpublished Results


VITA

John “Tod” Baker was born in Lake Charles, Louisiana on August 5\textsuperscript{th}, 1986. Tod attended high school at Alfred M. Barbe High School and graduated from this school in May 2005. He then attended Louisiana State University and Agricultural and Mechanical College in Fall 2005 and ultimately earned a Bachelor of Science Degree in Biochemistry at this university in May 2009. In Fall 2009, Tod began medical school at Tulane University School of Medicine in New Orleans, Louisiana. He spent two years at Tulane University School of Medicine and passed the STEP 1 USMLE (a major milestone in United States medical education). Tod then decided that he was more interested in scientific research, especially research involving biophysics. This led him to attend graduate school at Louisiana State University under the direction of Dr. Vince LiCata. Tod’s mother and father live in Lake Charles and Tod has an older brother who is a Physician’s Assistant in Buffalo, New York.