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The Glutamate Effect on the Functionality of Pol I DNA Polymerases

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by
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ABSTRACT

The enhancement of protein-DNA interactions in the presence of glutamate salts has been observed in numerous studies and has often been called "the glutamate effect". Klenow and Klentaq are the large fragment domains of the Type I DNA polymerases from *Escherichia coli* and *Thermus aquaticus*, respectively. Our lab has previously characterized the enhancing effect of potassium glutamate (KGlu) on the binding affinities of these polymerases to DNA versus that of potassium chloride (KCl). In this project, we investigate the effect of KGlu on the overall functionalities of Klenow and Klentaq.

Replacing KCl with KGlu in the reaction buffer for the polymerase chain reaction (PCR) extends the salt range that Taq polymerase remains functional in the DNA replication reaction. The highest salt concentration that yields PCR product in KCl is 100mM and in KGlu is 200mM. The effect on KGlu on the overall DNA replication reaction found in regular PCR was further reinforced by the use of qPCR method. The nucleotide incorporation rates of the two polymerases on activated calf thymus DNA was also investigated in the presence of increasing amounts of KCl and KGlu. Both enzymes incorporate more nucleotides in KGlu than in KCl up to 1000mM salt. The activity of Klenow is first activated by a small addition of salt and is inhibited at salt concentrations higher than 150mM while Klentaq is inhibited at all salt concentrations. Replacing KCl by KGlu in the solution buffer also resulted in a modest decrease in the melting temperature of duplex DNA at various salt concentrations.

It is thus found that the osmotic "glutamate effect" on polymerase binding carries over into the functional activity of Klenow and Klentaq polymerases at lower salt concentrations, while the effects of glutamate on polymerase binding at higher concentrations (> 1M) appears to be unrelated to the overall functionalities of Klentaq polymerase.

INTRODUCTION

DNA in all cells is constantly and faithfully replicated to meet the continual demand of cellular growth and survival. In all organisms, DNA replication is carried out by a family of enzymes called DNA polymerases. All DNA polymerases synthesize DNA in the 5'→3' direction and require the presence of primers and templates. The first step in the polymerase functional cycle is the binding of the enzyme to the primer-template DNA to form a binary complex. An incoming nucleotide (dNTP) is then added into the DNA-polymerase complex to form a ternary complex. The whole complex then undergoes a conformational change, and the 3'OH group of the primer performs a nucleophilic attack on the α-phosphate of the incoming dNTP to form a covalent bond. The resulting pyrophosphate is then released. The DNA-polymerase complex can then either dissociate, or it can bind another nucleotide and start the reaction cycle again. Those enzymes that can incorporate multiple nucleotides without releasing the DNA template are called "processive" enzymes (Joyce 2004).

Seven different families of DNA polymerases have been identified based on their phylogenic relationships: A, B, C, D, X, Y, and RT. The type I DNA polymerases from *Escherichia coli (*Pol I) and *Thermus aquaticus* (Taq polymerase)—the focus of this research study—belong to the A family polymerases. Pol I and Taq polymerases are not the main replicative enzymes in their bacteria; however, they replace the Okazaki fragments during lagging strand synthesis and repair damaged DNA (Lodish 2008). Pol I is a single polypeptide with 928 amino acids and a 103kDa molecular weight (Jovin 1969). Taq polymerase is also a single polypeptide with 832 amino acids and a molecular weight of 94kDa (Chien 1976). The Pol I and Taq polymerases share 38% sequence identity and are classified as homologs based on their amino acid sequence alignment (Lawyer 1989).

Both Pol I and Taq polymerases possess a 5'→3' nuclease domain, which is located at the N-terminus and is responsible for the removal of RNA primers from the Okazaki fragments during lagging strand synthesis, and a 5'→3' polymerase domain, which is located at the C-terminus and is responsible for adding incoming nucleotides onto the 3' end of the primer. Pol I has a functional 3'→5' exonuclease proofreading domain, which allows it to remove misincorporated nucleotides from the 3' end of the primer. The proofreading domain of Taq polymerase, on the other hand, does not possess catalytic activity. Thus, Taq is considered to be an error-prone polymerase and misincorporates a dNTP every 10^5-10^6 incorporations (Eckert and Kunkel 1990). However, Taq is still widely used as an important reagent in the polymerase chain reaction because of its extreme thermostability. The enzyme can retain its functionality at temperature as high as 95˚C, which is necessary to denature the template DNA in the replication reaction (Eckert 1990).

The removal of the 5'→3'nuclease domains from full length Pol I and Taq polymerases yield the large fragments Klenow (68kDa) and Klentaq (62kDa) (Barnes 1992 and Klenow 1970). X-ray crystal structures of Klenow and Klentaq show that their structures are highly similar, and the two proteins share ~49% sequence identity (Fig.1). The polymerase domains of Klenow and Klentaq polymerases have a common architectural feature that resembles an "open right hand" with "fingers", "thumb", and "palm" subdomains. The "fingers" subdomain binds to the single stranded portion of the template and to the incoming dNTP. The "thumb" subdomain binds the duplex region of the DNA. The active site residues of the enzymes lie in the palm subdomain and function to orient the primer strand and to carry out phosphodiester bond formation (Ollis et. al. 1985). The secondary structures of Klenow and Klentaq are also highly similar and contain predominantly alpha-helical structure.

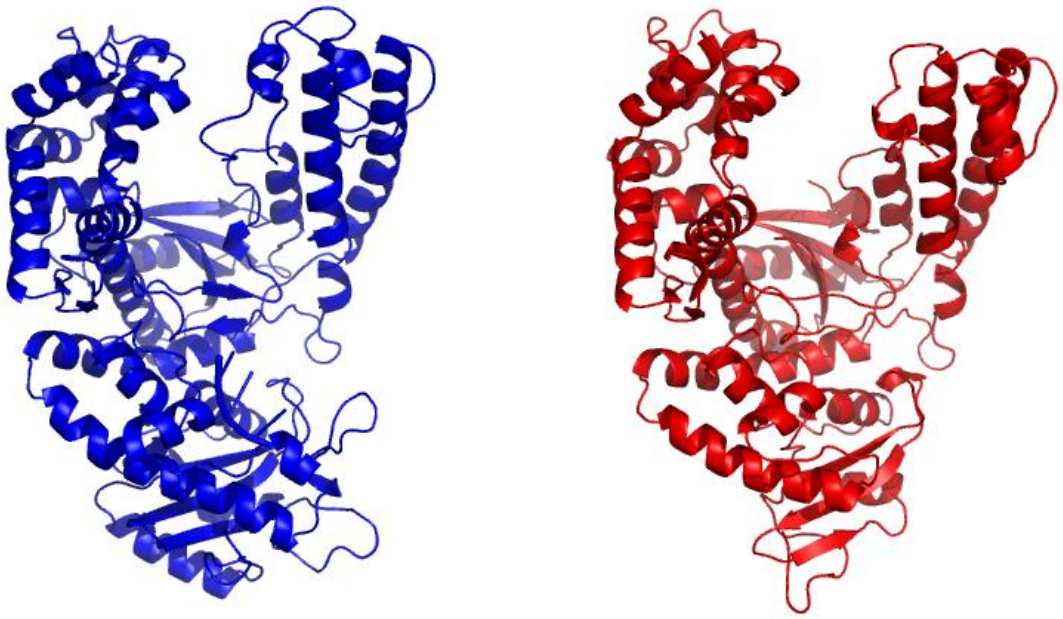

Figure 1: X-ray crystal structures of Klenow (left, Protein Data Bank ID# 1KFD-Beese et. al. 1993) and Klentaq (right, PDB ID# 1KTQ-Korolev et. al. 1995) represented in ribbon diagram.

Although Klenow and Klentaq share highly similar three-dimensional structures, their DNA binding properties are somewhat different. The two polymerases bind DNA with submicromolar affinities in very different salt concentration ranges. Klenow is much more salt resistant than Klentaq and can bind DNA with submicromolar K_d values at much higher KCl concentrations than Klentaq (appropriately an order of magnitude higher). Also, at any salt concentration, the binding affinity of Klenow to the same piece of DNA is ~150 times (~3kcal/mol) tighter than that of Klentaq. The binding of Klenow to DNA also releases ~50% more linked ions than Klentaq, which indicates the differences in the footprint of the two polymerases on DNA (Datta and LiCata 2003). The effect of temperature on the binding of Taq and Klentaq polymerases to DNA is also well-characterized. Despite the fact that Taq and Klentaq are thermophilic proteins, these two polymerases can bind DNA with high affinity at

temperatures as low as 5˚C. However, the DNA binding affinity of Taq/Klentaq is maximal at 40-50˚C (Datta and LiCata 2003).

Thermodynamic characterization of salt effects on protein-DNA interactions is well-studied. It is established that the binding of protein to DNA generally causes a net release of cations from the surface of negatively charged DNA molecules, which increases the entropy of the reaction and acts as a driving thermodynamic force for the binding between the two macromolecules (Record et. al. 1976). The equilibrium constant for the binding reaction of protein and DNA can be obtained as a function of salt. DNA-protein binding is highly favorable at low salt concentrations. However, as the salt concentrations increase, the reaction becomes less and less favorable. A log-log or ln-ln plot (d log (K_a)/d log [salt]) can be constructed, and the slope of this plot is predicted to be the number of cations released from the surface of the DNA molecules based on a basic Wyman linkage (Record et. al. 1976).

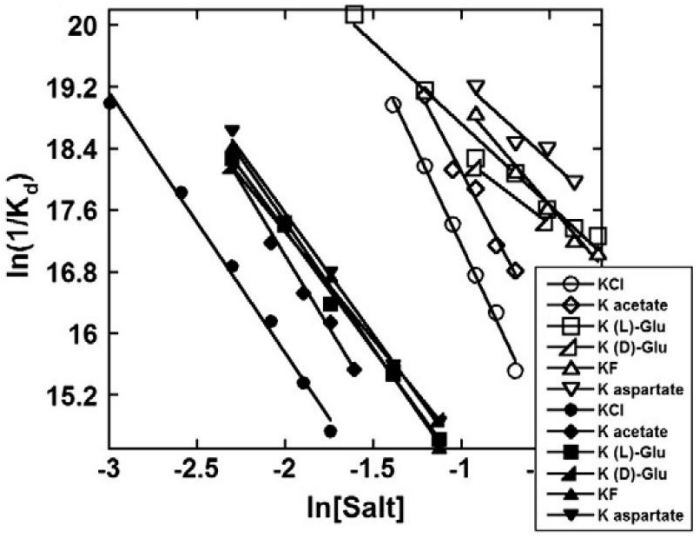

Figure 2: Salt linkages for Klenow (open symbols) and Klentaq (filled symbols) in the presence of: KCl, K-acetate, K-glutamate, K-aspartate, and KF

It has also been shown that differences in the types of anion in the solution can also affect the thermodynamics of protein-DNA binding. For example, Deredge et. al. have shown that varying the anion types can lead to changes in the binding affinity between DNA and polymerase. It is established that any particular salt concentration, the binding affinities of DNA to Klenow and

Klentaq polymerases increase in the following order: chloride < acetate < glutamate ~ fluoride ~ aspartate (Fig.2) (Deredge et. al. 2010).

In this thesis project, we are particularly interested in the effect of glutamate on interaction of protein and DNA because glutamate is a physiologically relevant ion and is often found accumulated inside *E.coli* cells to response to an external increase of salt. Compared to chloride ion, glutamate is also found to enhance the binding affinities and activities of several *E.coli* DNA binding proteins such as DNA polymerase III (Griep and McHenry 1989), and Ribonuclease III (Li et. al. 1993). Moreover, in glutamate salts, the binding and activity of these proteins are also extended to higher salt concentrations. These enhancement effects of glutamate on protein-DNA interactions have often been called "the glutamate effect". Deredge et. al also demonstrated the significant osmotic effect of glutamate ion in DNA-protein interaction. For example, replacing chloride by glutamate in the reaction solution markedly reduced the numbers of water molecules released upon DNA binding of both Klenow and Klentaq polymerases (~70% reduction) (Deredge et. al 2010).

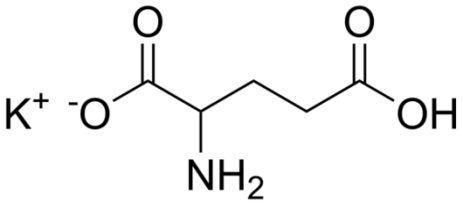

Figure 3: Formula structure of potassium glutamate

Furthermore, Deredge et. al. observed a rare occasion of reversal of the salt linkage that occurs at above 800mM for both Klenow and Klentaq through the use of salt-addition experiments (Figure 3). Deredge et. al. went on to show that inclusion of water release into the salt dependence of binding can quantitatively account for this apparent reversal of the classic salt effect. As salt concentration increases, the protein-DNA complex first dissociated due to increased ionic strength. However, when the salt concentrations are higher than 800mM in KGlu, the inhibition effect of ionic strength is offset by the enhancement of the osmotic effect,

which allows the protein-DNA complex to reform at salt concentrations as high as 2M. In KCl, without the favorable osmotic effect, the DNA and protein remain dissociated at high salt concentrations in KCl (Fig. 4) (Deredge et. al. 2010).

The thermodynamic binding of DNA and protein is only the first step of the DNA replication reaction. In this thesis, I ask if the favorable osmotic effect of glutamate on the binding of Klenow and Klentaq to DNA at both high salt and low salt concentrations is carried over to enhance other steps of the DNA replication reaction, such as nucleotide incorporation activity, thus making the overall reaction become more efficient, and possibly allowing DNA replication to occur at salt concentrations that were not previously reported. As such, the purposes of this research project are to:

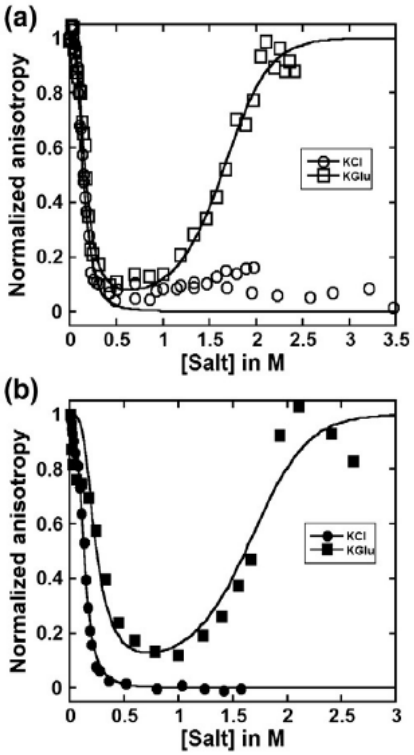

Figure 4: Salt-addition titration of (a) Klenow and (b) Klentaq showed a reversal of salt linkage in KGlu. Both polymerases bind DNA to saturation at low salt concentration. As the salt concentrations increased, the complex dissociated. However, at ~800mM KGlu, the polymerase rebind together and remain tightly bound up to salt concentrations as

1) Investigate whether the enhancing osmotic effect of glutamate on polymerase binding is carried over into the nucleotide incorporation activity both at low and high salt concentrations
2) Study the effect of glutamate on overall replication cycle carried out by Taq polymerase during the polymerase chain reaction.
3) Characterize the effect of glutamate on the melting temperature of duplex DNA compared with that of chloride.

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Proteins:

The proteins examined in this study were Klenow and Klentaq, the large fragments of *E.coli* DNA Pol I and Taq polymerase, respectively. The Klenow fragment used in this characterization is an exonuclease deficient D424A mutant (exo-minus Klenow fragment) (Derbyhire et. al. 1988). The clone of exo$^-$ Klenow was a gift from Catherine Joyce at Yale University. The clone of Klentaq was constructed by Wayne Barnes and obtained from the American Type Culture Collections (Barnes 1992). The protein concentrations were measured using optical density measurement at 280nm on a Cary 100 spectrophotometer (as described previously-Datta and LiCata 2003).

3.1.2 Nucleotide Incorporation Assay

Radioactive [α-32P] dATP was obtained from Perkin-Elmer (Waltham, Massachusetts). Sheared calf thymus DNA was purchased from Sigma-Aldrich (St. Louis, MO). DE81 filters were obtained from Whatman (Piscataway, New Jersey).

3.1.3 Polymerase Chain Reaction (PCR)

The template used for PCR was the 5874 base pairs plasmid pXS106, encoding exo$^-$ Klenow fragment. Go Taq polymerase and dNTPs were obtained from Promega Corporation (Madison, WI). Primers were obtained from Integrated DNA Technologies (Coralville, IA). The sequence of the forward primer is CCGTTGCCAAAAGTGATTCTG (T_m=54.8˚C). The sequence of the reverse primer is GTTACTGCCTGGTGATAAGAGG (T_m=54.9˚C). SYBR green I nucleic acid gel stain (SYBR green) was purchased from Invitrogen (Eugene, OR).

3.1.4 Quantitative PCR (qPCR)

Reference dye for qPCR (100x) was purchased from Sigma-Aldrich (St. Louis, MO). Primers and DNA template were the same as PCR experiment.

3.1.5 Oligonucleotides for DNA Melting Experiments

Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Oligonucleotides concentrations were determined spectrophotometrically using the extinction coefficient at 260nm provided by the company. Double stranded DNAs were prepared by combining equal volumes of 20μM solution of each strand. The solution was heated for 5 minutes at 94˚C to eliminate any secondary structure that each single strand DNA might have. The two strands were then annealed as the solution was cooled down to room temperature.

The sequence of the 20 mer-double stranded DNA used in the DNA melting experiments was

5'-TCGCAGCCGTCCAAGGTTT-3'

3'-AGCGTCGGCAGGTTCCAAA-5'

3.2 METHODS

3.2.1 Nucleotide Incorporation Assay

The effects of increasing salt concentration and of various anion types on the polymerization activities of Klenow and Klentaq were studied using a nucleotide incorporation assay. Radioactive [α-32P] dATP was used to monitor the amount of nucleotides incorporated by either Klenow or Klentaq into sheared calf thymus DNA in a 30-minute reaction. The assay was carried out at 35˚C for Klenow, a mesophilic protein and at 50˚C for Klentaq, a thermophilic protein.

The total volume of the reaction was 25μL and included the following components

1) 10nM sheared calf thymus DNA
2) 1μM protein

3) 200μM of dATP, dTTP, dCTP, and dGTP
4) 100μCi/mL of [α-32P] dATP
5) Reaction buffer: 10mMTris, 5mM $MgCl_2$, 0.1%Tween 20, pH 7.9
6) Either KCl or KGlu at various concentrations

The reaction components were first equilibrated at the appropriate temperature using a water bath. The reaction was started when protein was added into the reaction solution. After 30 minutes, the reaction was stopped by the addition of 25μL of 300mM EDTA. Next, 10μL aliquots were taken from the reactions and blotted onto Whatman DE81 filters. After all the newly polymerized DNA was deposited on the filters, the excess radioactive [α-32P] dATP was removed in three successive, five minute-washes in 300mM sodium phosphate pH 7(with gentle swirling at 30-second intervals). Next, the filters were washed for 2 minutes in 70% ethanol and then air-dried. The amount of radioactive DNA on the filters was counted using a Tri-Carb 2900TR Liquid Scintillation analyzer from Packard Bioscience (Perkin-Elmer).

To calculate the amount of nucleotides incorporated by Klenow and Klentaq polymerases in a 30-minute reaction, background and total radioactivity readings were also measured. In background samples, buffer was added into the reaction volume instead of protein. In the total radioactivity samples, excess [α-32P] dATP was not washed from the filters.

The amount of radioactive [α-32P] dATP incorporated in each sample equals:

[Sample count (cpm)-Background count (cpm)]/total count (cpm)

The amounts of nucleotides incorporated by the enzyme (in pmoles unit) were assumed to be 4 times of the amount of dATPs incorporated and was calculated based on this formula:

pmoles of dNTPs = amount of radioactive [α-32P] dATP incorporated

*** 200μMdATP* 25μL*4**

The steady state rate of nucleotide incorporation was determined based on this formula:

Steady state rate= dNTPs incorporated/ time.

3.2.2 Polymerase Chain Reaction (PCR)

Amplification reactions were carried out under the following conditions: 10mM Tris-HCl pH 8, 2.5mM $MgCl_2$, 1 μM forward primer, 1 μM reverse primer, 0.02ng/μL template, 200μM dATP, dTTP, dCTP, and dGTP, 0.025units/ μL Go Taq polymerase, and variable amounts of KCl or KGlu (salt concentrations varied from 0-2M).

PCR was conducted in GeneAmp PCR system 9700 instrument from Applied Biosystems (Foster City, CA). DNA templates were first subjected to a denaturation step for 10 minutes at 95˚C. The thermal cycling conditions were as follows: denaturation at 95˚C for 1 minute, annealing at 55˚C for 1 minute, and extension at 72˚C for 2 minutes. All PCR was carried out for 30 cycles of amplification. Finished products were analyzed by polyacrylamide gels stained with SYBR green dye, visualized on a UV transilluminator and documented by digital photography.

3.2.3 Quantitative PCR (qPCR)

Most qPCR reactions are carried out in a pre-made master mix where all the necessary components (dNTPs, $MgCl_2$, Taq polymerase, reaction buffer, and enhancements) are supplied as a kit. The only things needed to be added are the DNA template and primers. However, the purpose of our project is to investigate the effect of salt on the DNA replication reaction. Thus, we had to prepare a "homemade" mix, which included 2.5 mM $MgCl_2$, 200μM of dATP, dTTP, dCTP, and dGTP, 0.02μM forward primer, 0.2μM reverse primer, 8% Glycerol, 3% DMSO, 0.1x reference dye, 0.125x SYBR dye, 0.025units/μL Go Taq polymerase, 0.004ng/μL template, and variable amounts of KCl or KGlu. The relative fluorescence signal observed in qPCR is the ratio

between the fluorescence level of SYBR dye and that of the reference dye. Precaution needs to be used when carrying out qPCR to prevent cross contamination between samples.

qPCR was conducted in a Model 7500 Real-Time PCR System instrument from Applied Biosystems (Foster City, CA). DNA templates were first subjected to a denaturation step for 10 minutes at 95˚C. The thermal cycling conditions were as follows: denaturation at 95˚C for 30 seconds, annealing/extension at 60˚C for 1 minute. All qPCR was carried out for 40 cycles of amplification.

3.2.4 DNA melting temperature

DNA melting studies were conducted in 10mM potassium phosphate buffer (pH 8) with the various concentration of either KCl and KGlu. The final concentration of 20-mer duplex DNA was 250nM. Thermal denaturation of DNA duplex was monitored at 260nm in a Cary 100 spectrophotometer connected to a water bath. Absorbance readings were recorded every 1˚ increase in temperature.

Thermal denaturation curves were fitted to a modified form of the van't Hoff equation, which fits the native and denatured baseline and the transition region to obtain the Tm value for denaturation (Ramsay and Eftink 1994).

$$\Delta\varepsilon = (m_n\mathrm{T} + b_n) + \frac{(m_d\mathrm{T} + b_d)e^{\frac{-\Delta H\left(1-\frac{T}{T_m}\right)}{RT}}}{1 + e^{\frac{-\Delta H\left(1-\frac{T}{T_m}\right)}{RT}}}$$

Here, m_n, m_d, and b_n, b_d are the slope and intercepts of the native and denatured state base lines respectively. T is the temperature, and ΔH is the enthalpy of the denaturation reaction. Data were fitted using the program in Kaleidagraph from Synergy Software (Reading, Pennsylvania).

RESULTS

1) Nucleotide incorporation assay

In order to investigate the effect of glutamate on the polymerization activity of Klenow and Klentaq, radioactive nucleotide incorporation reactions were carried out in both KCl and KGlu at various salt concentrations. The amount of total nucleotides incorporated by the two enzymes in 30-minute reactions and their steady state rates were recorded.

The reactions were carried out at a temperature close to optimal for Klenow (35˚C) and Klentaq (50˚C). The amount of nucleotides incorporated by Klenow in a 30-minute reaction was consistently higher than Klentaq at all salt concentration as seen in Figure 5. The polymerization activity of Klenow was activated by a small addition of salt before the onset of salt-induced inhibition while Klentaq was monotonically inhibited as salt increased. In a 30-minute reaction, Klenow and Klentaq incorporated the most nucleotides (1278 pmoles/1 μM enzyme and 112 pmoles/1 μM enzyme) at 150mM KGlu and 0 salts, respectively (Table 1).

Both Klenow and Klentaq incorporated more nucleotides in a 30-minute reaction in KGlu than in KCl. At 400mM KCl, the activities of both polymerases were markedly attenuated. The steady state rate at 400mM KCl was ~2% of the fastest rate in Klenow and 1.6% of the fastest rate in Klentaq. On the other hand, the activities of both polymerases were maintained to much higher salt concentration in KGlu. At 1M KGlu, the steady state rate of Klenow was still 8.2% of the fastest rate and the steady state rate of Klentaq was ~10% of the fastest rate.

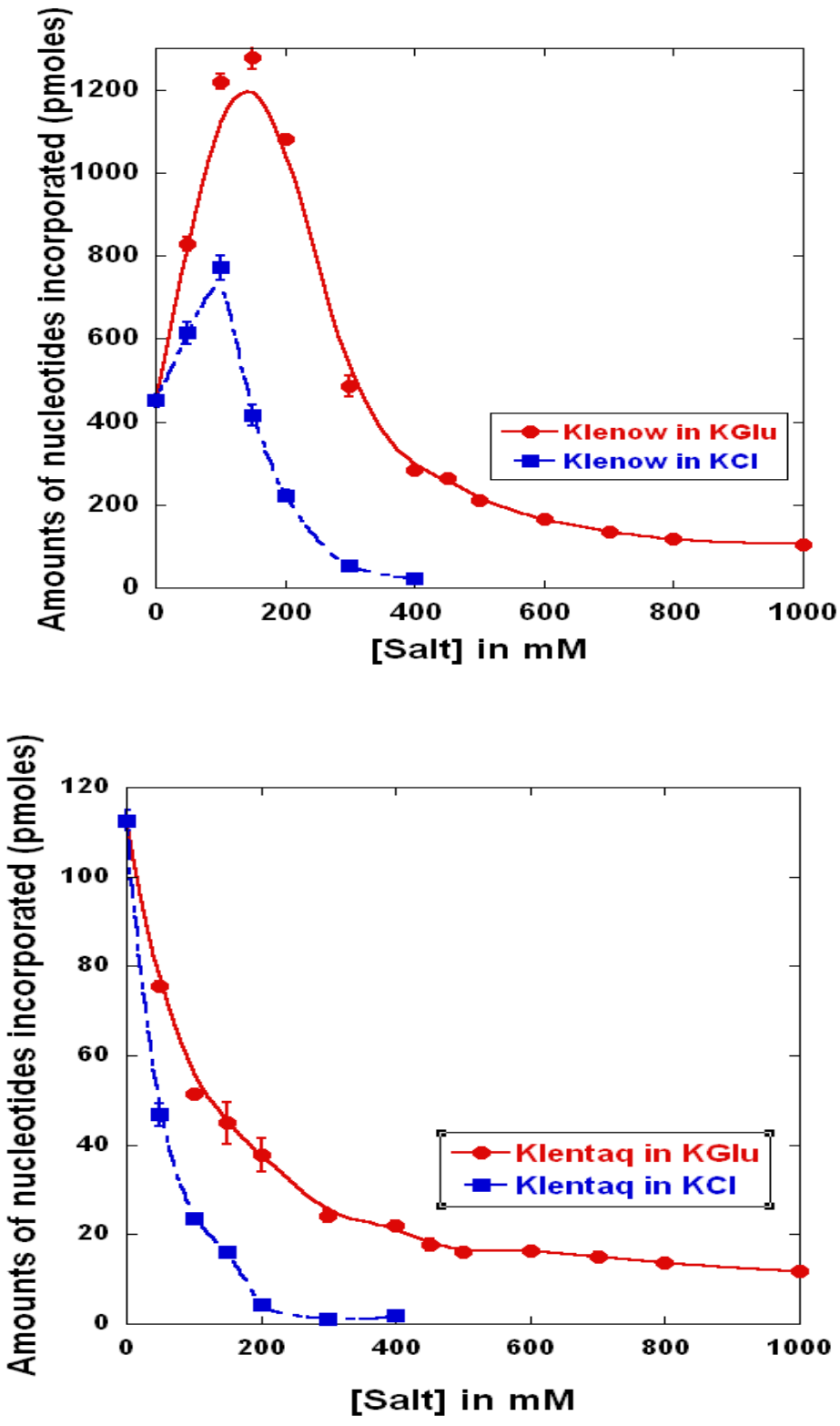

Figure 5: Amounts of nucleotides incorporated by Klenow (A) and Klentaq (B) into sheared calf thymus DNA as function of salt types and concentrations in KCl and KGlu. Lines are simple extrapolations for ease of visualization.

Table 1: Steady state nucleotide incorporation kinetics of Klenow with sheared calf thymus DNA. The amount of nucleotides incorporated by 1 μM enzyme in a 30 minute-reaction is reported.

Salt concentration (M)	Nucleotides incorporated in Kglu (pmoles)	Nucleotides incorporated in KCl (pmoles)
0	451.2 ± 26.3	451.2 ± 26.3
0.05	827.9 ± 29.9	613.9 ± 45.1
0.1	1219.5 ± 32.6	772.3 ± 51.2
0.15	1278.4 ± 51.7	415.7 ± 43.0
0.2	1081.7 ± 17.6	221.6 ± 23.1
0.3	485.6 ± 44.2	52.4 ± 1.5
0.4	283.2 ± 17.9	21.7 ± 1.87
0.45	264.9 ± 9.2	
0.5	212.0 ± 16.9	
0.6	166.6 ± 4.2	
0.7	135.3 ± 9.8	
0.8	117.1 ± 2.7	
1	105.3 ± 2.0	

Table 2: Steady state nucleotide incorporation kinetics of Klentaq with sheared calf thymus DNA. The amount of nucleotides incorporated by 1 μM enzyme in a 30 minute-reaction is reported.

Salt concentration (M)	Nucleotides incorporated in Kglu (pmoles)	Nucleotides incorporated in KCl (pmoles)
0	112.6 ± 4.1	112.6 + 4.1
0.05	75.4 ± 1.9	46.7 ± 4.6
0.1	51.5 ± 1.1	23.4 ± 1.0
0.15	44.9 ± 5.1	16 ± 1.4
0.2	37.8 ± 4.6	4.1 ± 1.2
0.3	24.1 ± 2.5	1.1 ± 0.1
0.4	21.9 ± 1.7	1.8 ± 0.1
0.45	17.7 ± 2.4	
0.5	16.1 ± 0.6	
0.6	16.4 ± 1.1	
0.7	15.1 ± 1.5	
0.8	13.7 ± 0.7	
1	11.7 ± 0.6	

2) Polymerase Chain Reaction

To further examine the functional behavior of Klentaq in KGlu, we performed PCR as a function of salt types and concentrations. DNA product was obtained in both KGlu and KCl at low salt concentrations up to 100mM. No product was obtained in KCl at any salt concentration higher than 100mM. In KGlu, the last salt concentration that yielded detectable product was extended to 150mM salt when no PCR enhancers were added (Fig. 6A). When 8% Glycerol, 3% DMSO were added as enhancers, DNA amplification was observed up to 200mMKGlu. Enhancers, however, did not alter the result with KCl. No DNA was detected at any concentration higher than 200mM in KGlu, even in between 1.8M and 2M, the salt range that binding between DNA and polymerases were shown to exist (Fig. 6B).

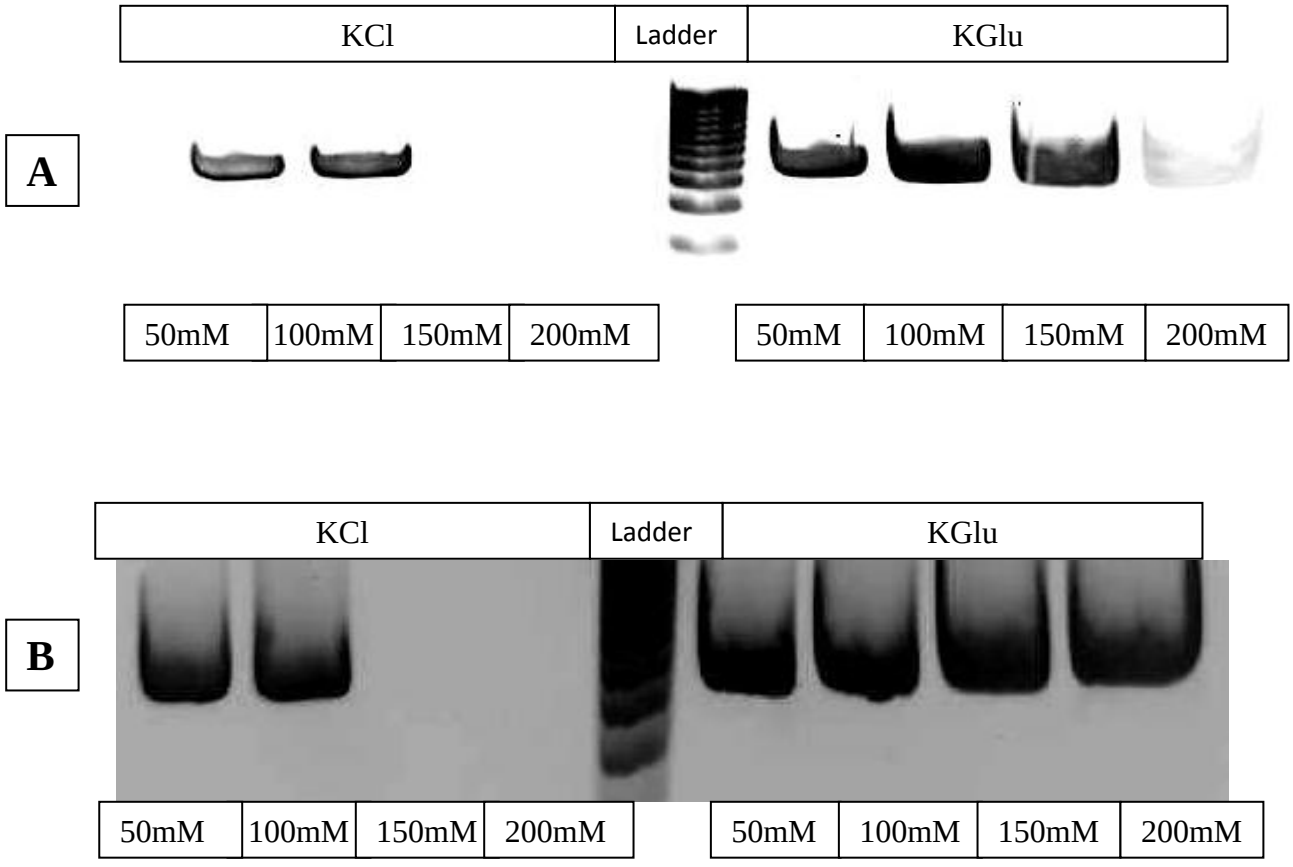

Figure 6: PCR amplification product in various salt concentrations of KGlu and KCl without (A) and with (B) glycerol and DMSO enhancers

3) Quantitative polymerase chain reaction (qPCR)

qPCR was carried out to further characterize the effect of glutamate on the overall DNA replication reaction performed by Taq polymerase. qPCR, also known as real time-PCR, measures PCR amplification as the reaction occurs. Therefore, it allows the detection of not only the end product of the replication reaction but also the gradual accumulation of DNA product as the reaction progresses. qPCR utilizes a fluorescent reporter molecule, and as the quantity of target product increases, so does the amount of fluorescence emitted from the fluorescent reporter. Thus, an increase in reporter fluorescence signal is directly proportional to the number of product strands generated.

There are two commonly used types of fluorescent reporter molecules: TaqMan Probe system and SYBR green dye. In TaqMan based detection, a specific probe is designed for each target, and either appropriate hybridization between probe and target or displacement of the probe from the target is required to generate fluorescence signal. In comparison, SYBR green dye binds all double stranded DNA products. Thus, TaqMan is more specific and can eliminate false positives whereas SYBR is easier to use and costs less. In this project, we utilized SYBR green dye because as Figure 6 shows, there are no non-specific products formed during this amplification.

There are two different types of qPCR: absolute quantification and relative quantification. Absolute quantification determines the input copy number by constructing a standard curve via serial dilution of a known DNA concentration. Relative quantification compares the PCR signal of an amplified target transcript in a treatment group to that of an untreated control. In this experiment, we wanted to compare the effect of KGlu to that of KCl on the DNA replication reaction performed by Taq polymerase. Thus, we chose to perform the relative quantification

method. We used the same initial DNA template and primer concentrations in all experiments and measured the increase of DNA product in both KGlu and KCl

In qPCR, again, we only observed DNA product up to 100mM KCl and up to 200mM KGlu. Initial results also showed that the fluorescence level at the plateau phase decreased as the salt concentration increased. Thus, the ranking of fluorescence levels were as follow: 50mM>100mM>150mM>200mM. Initial results led to an impression that more products were made in KGlu than in KCl and that lower salt concentrations produced slightly more product compared to higher salt concentrations (Fig. 7). However, it has been previously shown that increasing salt concentrations can interfere with the hydrophobic binding of SYBR binding to DNA (Zipper et. al. 2004), and thus could cause differences in the fluorescent levels of SYBR green in different salt types and salt concentrations. Based on the result from the regular PCR experiment (Figure 5), we suspected that changes in binding affinity of SYBR were the reason for the varying fluorescence signal at plateau values in different salt types and concentrations. To investigate whether this was the case, we performed normal qPCR reactions in KGlu at various concentrations, and when the reaction finished, additional KGlu was added into each well to bring the final salt concentration in all wells to 200mM. The fluorescence level was then determined again. As hypothesized, the plateau fluorescence level became equal for all samples. This proved that replacing KCl by KGlu only extended the salt range that the polymerases still remain functional and can replicate DNA, but that KGlu does not help increase the total quantity of DNA product made (Figure 8).

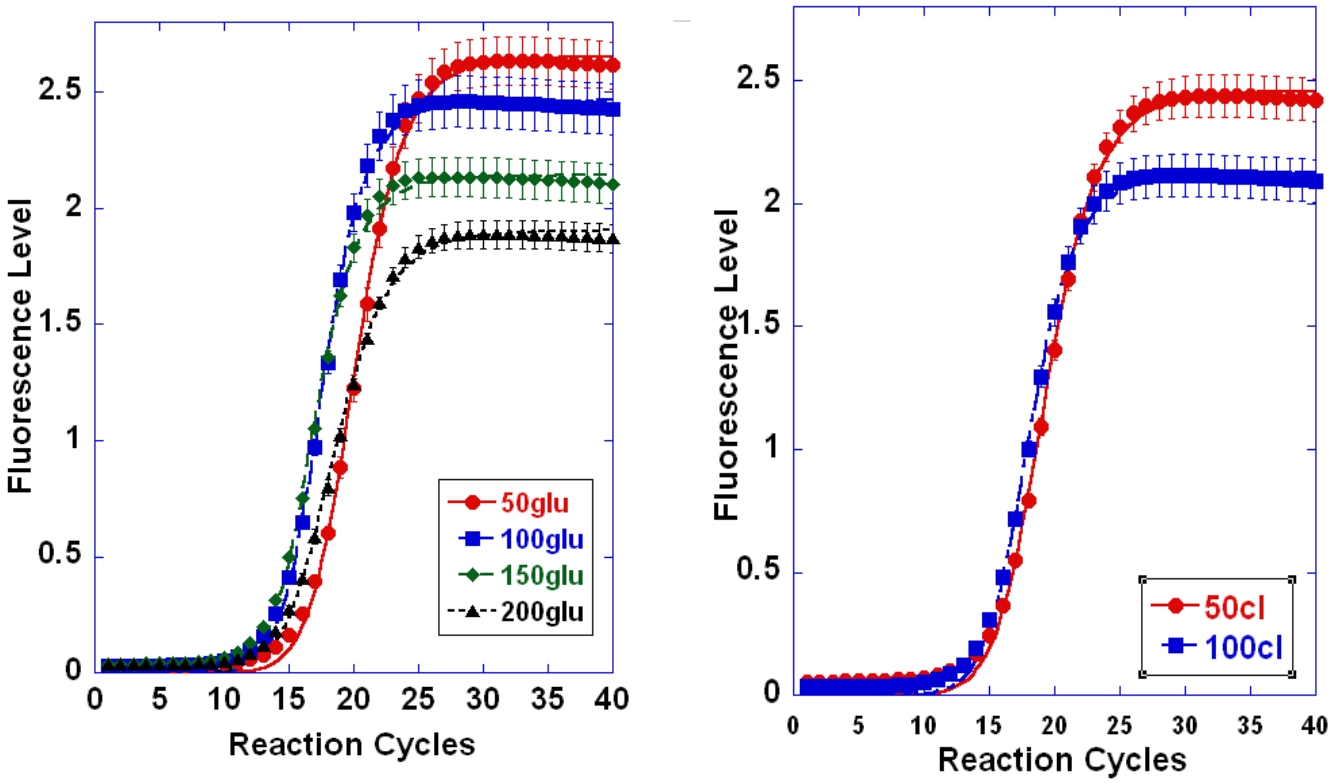

Figure 7: Overrall replication reaction in qPCR. Relative fluorescence value is plotted against cycle number and the baselines as well as the plateau values of both reactions are shown

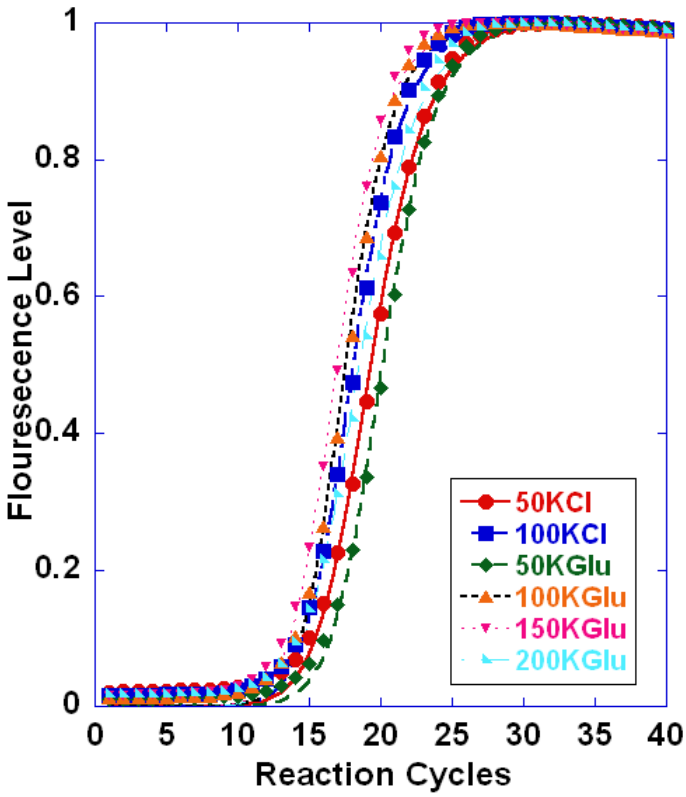

Figure 8: Normalized floresecence signal of qPCR reaction in various salt types and concentrations after adjusting the salt concentrations in all samples

One commonly obtained parameter in qPCR reaction is the cycle threshold of the reaction (C_t value). C_t is the number of the first cycle where the fluorescence signal exceeds the background level. C_t values are inversely proportional to the amount of the initial target DNA added in the samples (the more target DNA present, the fewer cycles it takes to bring the fluorescence level pass the threshold). The values of C_t values obtained in the qPCR reactions performed in KCl and KGlu in various salt concentrations are presented in Table 4.

Table 4: The values of cycle threshold (C_t) of qPCR in various concentrations of KCl and KGlu. The numbers are the averages of 9 separate amplifications.

Salt concentrations (mM)/Type	KGlu	KCl
50	16.49 $\pm$ 0.25	16.05 $\pm$ 0.04
100	15.13 $\pm$ 0.10	15.50 $\pm$ 0.09
150	14.96 $\pm$ 0.07	
200	15.81 $\pm$ 0.29	

qPCR done in 150mM KGlu had the lowest C_t value among the salt concentrations examined. However, the difference was relatively subtle, and the C_t values of the qPCR reaction done in 100mM KCl and KGlu, 150mM KGlu, and 200mMKGlu are relatively close to one another at around cycle 15. C_t values of qPCR done in 50mMKGlu and KCl, interestingly, both had higher C_t values, at ~cycle 16.

4) <u>The effect of salt on the melting temperature of duplex DNA.</u>

Because nucleotide incorporation persists into much higher salt concentrations than does PCR (e.g. Figure 5 vs. Figure 6, 7, and 8), it appears that salt detrimentally affects some other processes in PCR (other than nucleotide incorporation). One such process we examined was the DNA melting temperature.

Melting temperatures of DNA duplex in increasing concentrations of KCl and KGlu were also measured to insure that the absence of PCR product at high salt concentrations were not due to the DNA denaturation step of the replication cycle. As the salt concentrations increased, the melting temperatures of the DNA duplex also increased in both salts. However, the increment was relatively small across a large salt concentrations range. The melting temperature of DNA only increased by ~10°C in both KCl and KGlu when salt concentrations were raised from 50mM to 2M. Interestingly, replacing KCl by KGlu decreased the melting temperatures of DNA duplex by an average of 4°C (Figure 9 and Table 3)

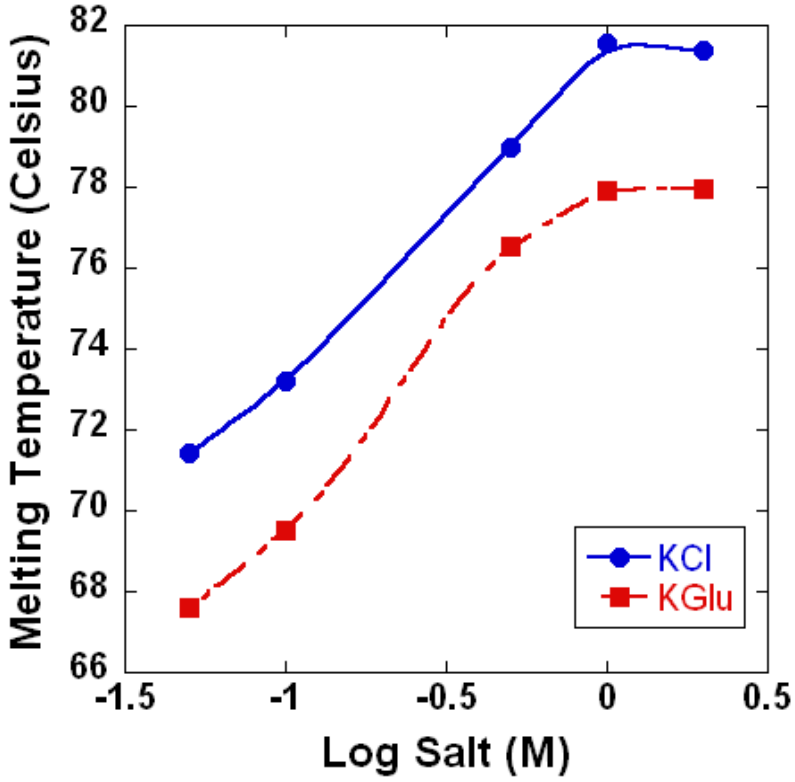

Figure 9: Comparison of melting temperature of 20mer duplex DNA in various concentrations of KCl and KGlu.

Table 3: The melting temperature of a 20mer duplex in various concentrations of KCl and KGlu

Salt concentration (M)	T_m (°C) in KCl	T_m (°C) in KGlu
0.05	71.4	67.6
0.1	73.2	69.5
0.5	79	76.5
1	81.6	77.9
2	81.4	77.9

DISCUSSION

In this project, we investigated the effect of KGlu versus that of KCl on the overall functionality of Type I DNA polymerases. We examined the effect of salt types and concentrations on (1) the nucleotide incorporation activities of both Klenow and Klentaq polymerases, (2) the overall PCR reaction by Taq polymerase, and (3) the melting temperature of duplex DNA.

The nucleotide incorporation activities of both Klenow and Klentaq were studied as a function of different salt types and concentrations. The enhancing osmotic effects previously observed in the binding of Klenow and Klentaq to DNA were carried over to their nucleotide incorporation activities as both Klenow and Klentaq incorporated more nucleotides in KGlu than in KCl across the entire salt range of the study (0-1000mM). This could be due to the tighter binding of the two polymerases to DNA in KGlu than in KCl. Also, at any particular salt concentration, Klenow consistently incorporated more nucleotides than Klentaq, and thus, had higher steady state rates of nucleotide incorporation. Datta et. al. previously showed that Klenow binds DNA ~150 folds tighter than Klentaq at all salt concentrations (Datta and LiCata 2003). Because Klenow and Klentaq are both processive enzymes, the tighter binding between the polymerase and the DNA template might enable Klenow to stay on the DNA template longer and incorporate more nucleotides before releasing it.

As the salt concentration increased, the incorporation activities of both Klenow and Klentaq decreased in both KGlu and KCl. Klentaq worked best at lower salt concentrations as the steady state rate of the enzyme consistently decreased as the salt concentration increased. The incorporation activity of Klenow, on the other hand, exhibited biphasic behavior. It first seemed to be activated by a small addition of salt (in both KGlu and KCl) as the steady state rate of

incorporation increased as the salt concentration increased up to 150mM and then decreased at concentrations >150mM. It was previously shown that the $MgCl_2$ linkage plot for Klenow-DNA binding displayed a similar pattern: a small uptake of linked Mg^{2+} at low $MgCl_2$ concentrations and a net release of 1.2 Mg^{2+} at $MgCl_2$ concentrations higher than 10mM (Datta and LiCata 2003). Thus, this is not the first time that Klenow has exhibited biphasic salt dependence behavior in its activity. Therefore, it is likely that Klenow requires a small uptake of salt to reach its optimal nucleotide incorporation activity.

Nucleotide incorporation data indicated that Klentaq remains functional and can incorporate low but detectable numbers of nucleotides across a very large range of KGlu (up to 1M). Thus, it was expected that we would be able to extend the salt range that Taq polymerase can perform the replication reaction in PCR. However, the highest salt concentration that PCR product was obtained in was 200mM KGlu.

The effect of salt types and concentrations on the overall DNA replication reaction carried out by Taq polymerase were also investigated using qPCR. The initial results from qPCR illustrated that more DNA was made in KGlu than in KCl and that the amount of DNA product made decreased as the salt concentration increased in both KGlu and KCl. However, these variations were later found to be caused by the difference in binding affinity of SYBR green dye to double stranded DNA because the fluorescent signals all dropped to the same levels when the salt concentrations in all samples were adjusted to the same levels after amplification occurred.

Thus, as salt is increased we observed a gradual decrease in nucleotide incorporation activity, but a sudden disappearance of PCR product. The reason is that if one step of the overall replication cycle is inhibited, the whole PCR reaction is inhibited. Replacing KCl by KGlu in the PCR reaction buffer does, however, extend the salt concentration range that yielded

detectable DNA product in PCR to at least 150mM salt. Interestingly, this salt concentrations range was extended slightly more, to 200mM KGlu, when 8% Glycerol and 3% DMSO were added to the reaction mix as enhancers. As the salt concentration increases, the melting temperature of DNA template also increases. Thus, DMSO and glycerol might enhance the amplification reaction by lowering the melting temperature of template DNA and allow the replication reaction to occur (Wang et. al. 1993), but they do not enhance the reaction in KCl.

A major question is: Why does the discrepancy between nucleotide incorporation versus PCR exist? Below are the steps of the kinetic mechanism of nucleotide incorporation by DNA polymerase I on primer template DNA:

1) Polymerase binds to primer template DNA to form a binary complex
2) Binding of incoming dNTP to the fingers domain of the polymerase
3) Conformational change of the complex occurs to prepare for the nucleophilic attack to take place (dNTP is now presented to the polymerase active site)
4) 3'OH on the primers perform nucleophilic attack on the alpha phosphate of the incoming dNTP
5) The complex undergoes another conformational change, and pyrophosphate is released
6) The binary complex can either dissociate or translocate by a base and binds another nucleotide to enter another incorporation cycle.

We were able to detect the nucleotide incorporation activity of Klentaq at salt concentrations as high as 1M KGlu, which proved that the enzyme was still functional and could perform all six steps above. Several explanations can be used to explain the absence of PCR product at salt concentrations as low as 250mM KGlu or 150mM KCl. A step that occurs in PCR but does not occur in the nucleotide incorporation assay is the hybridization of primer and DNA template.

We can speculate that high salt concentration (e.g. 250mM KGlu) could prevent this step from happening and thus inhibit the PCR reaction. But data (Figure 9) showed only a modest shift in the T_m for primer-length DNA annealing at elevated salt concentrations, which appeared to rule out this possibility. Another possible reason for the absence of DNA product at these salt concentrations is that the nucleotide incorporation rate of Taq polymerase is reduced at high salt concentrations, thus, the enzyme cannot add nucleotides as fast as it can in the lower salt environment (the steady state rate was markedly reduced). The nucleotide incorporation assay was done in a 30 minute reaction while the extension step in PCR was only done in 1 minute. So if we increase the extension step in the PCR reaction, it is possible that we can obtain DNA product. We have not yet tested this possibility.

Although Figure 8 shows that primer binding should not be greatly perturbed by salt, another reason could have been an increase in melting temperature of the amplified DNA template in such high salt concentrations preventing it from being denatured, which would inhibit all other steps of the replication reaction. The melting temperature of any DNA duplex longer than 50 nucleotides can be estimated from this equation:

Tm= 81.5 °C + 16.6*log10 ([Na+]/(1 + 0.7[Na+])) + 0.41(%G+C)-0500/L (Wetmur 1991)

Here [Na+] is the concentration of the monovalent cation in the solution, and L is the length of the plasmid to be amplified. Our plasmid is 5874 nucleotides long and has 47.6% GC. According to this calculation, when the salt concentration is increased from 0.05mM to 2M, the melting temperature of the plasmid increases from 80 ˚C to 103˚C, which is significantly higher than the standard denaturation temperature 95˚C. This possibility also has yet to be tested.

Although we did not obtain PCR product at KGlu concentrations higher than 200mM, it does not necessary mean that DNA replication reaction cannot occur at these concentrations. As we

have shown in this thesis and results from Deredge et. al., both DNA binding and nucleotide incorporation activity of Klentaq occur at KGlu concentrations much higher than 200mM. Therefore, it is likely that we could obtain PCR product at those concentrations, if we identify and eliminate the salt inhibition effect on the other steps of the PCR. As mentioned above, since the steady state rate of nucleotide incorporation of Klentaq polymerase is greatly reduced at 300mM KGlu compared with that at 200mM KGlu (~36% reduction) or with the highest steady state rate (~79% reduction), it is likely that the extension step of the PCR cycle needs to be longer for PCR product to be obtained at higher salt concentrations. As also mentioned above, the temperature of the denaturation step can also be a potential barrier for PCR to occur at higher salt concentration. Higher temperature can be used in the denaturation step to facilitate the denaturation of DNA. However, if the temperature is raised too high, the activity of Taq polymerase could be affected because the enzyme's half-life can be reduced from 120 minutes to 5 minutes when the temperature of the denaturation step is raised from 92.5°C to 97.5°C. Therefore, additives such as 1,2-propanediol and trehalose, which were previously shown to help decrease the DNA melting temperature (Horáková et. al. 2011) could be added to counteract the possible large increase in template denaturation temperature.

In this project, we find that the enhancing "glutamate effect" on DNA-polymerase binding carries over into nucleotide incorporation activity of Klenow and Klentaq polymerases, resulting in increased activity of both enzymes in KGlu compared with KCl up to 1M salt. However, the osmotic effects of glutamate on polymerase binding at higher concentrations (> 1M) appear to be unrelated to the overall functionalities of Klentaq polymerase. In PCR, replacing KCl by KGlu in the reaction buffer extends the salt concentrations range that yields detectable PCR product to 200mM compared with 100mM in KCl. Also, the melting temperature of duplex DNA is

slightly lower in KGlu than in KCl. We find that Klentaq remains functional and can perform nucleotide incorporation out to at least 1M KGlu while PCR is curtailed above 200mM KGlu; thus, these data indicate that while KGlu enhances polymerase function to high concentrations, it apparently inhibits some other steps of the PCR cycle which we have yet to identify.

While the enhancing effect of glutamate on the binding of protein to DNA has been studied for some time, the effects of glutamate on the function of DNA-processing enzymes have been far less examined. The works in this thesis are a first step in understanding how glutamate enhances the functionality of DNA polymerase.

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