Kinetic Analysis of Templated and Non-Templated Nucleotide Addition by Yeast tRNA$^{\text{His}}$ Guanylyltransferase

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ABSTRACT

The central dogma of DNA and RNA replication invokes the widely accepted rule of 5'-3' synthesis of nucleic acids. The discovery of the tRNA$^{\text{His}}$ guanylyltransferase (Thg1), which adds nucleotides to nucleic acid substrates in the 3'-5' direction, is the first exception to the rule. Since the reactions catalyzed by Thg1 are biochemically unprecedented, determination of the mechanism by which Thg1 catalyzes 3'-5' nucleotide addition is of great interest. Previous work in yeast Thg1 (yThg1) identified two residues that were involved in the adenylylation step of the 3'-5' addition reaction. The structure of yThg1 is not known, but the recently published high resolution crystal structure of the human Thg1 homolog inspired us to examine the analogous residues in the context of human Thg1 (hThg1) (Hyde et al., 2010). Our data suggests that similar to yThg1, K44 is a key player in binding the nucleotide (NTP) that is being added, while N158 may be involved in the chemistry of the adenylylation used to activate the tRNA.

Saccharomyces cerevisiae Thg1 incorporates nucleotides in both template-dependent and template-independent reactions – two fundamentally distinct activities (Jackman & Phizicky, 2008). To understand the biochemical basis for these two activities we measured the kinetics of specific steps during Thg1 catalysis. Rates of incorporation of each ribonucleotide across from adenosine (A$_{73}$), cytidine (C$_{73}$), and guanosine (G$_{73}$) were measured under single turnover conditions using an assay that specifically isolates the nucleotidyltransfer step. Our data strongly suggests that Thg1 displays two kinetically distinct mechanisms where the 73$^{\text{rd}}$ nucleotide in the tRNA substrate is critical in determining the mode of catalysis. While Thg1 demonstrates unusual kinetics for tRNA substrates containing an adenosine at the 73$^{\text{rd}}$ position (A$_{73}$), it displays characteristics and behaves very similar to a canonical replicative polymerase with
substrate tRNAs containing other nucleotides at that position. Finally, we were able to show that Thg1 incorporates useful nucleotide analogues with moieties such as dyes and biotin at the 5' position, which will be implemented to further study the novel mechanisms employed by the enzyme.
INTRODUCTION

Discovery and elucidation of novel systems is often the first step in formation of new rules, subgroups, classes, and even contradictions to existing dogma. Work of such nature is at the forefront process of modern scientific research. In 1958, Kornberg’s *Enzymatic Synthesis of Deoxyribonucleic Acid* presented a novel enzyme capable of replicating DNA. A half of a century later numerous contributing discoveries have proved, expanded, and posed new questions with possible exceptions to the initial discovery.

Until recently, all DNA and RNA polymerases were believed to act in the 5' to 3' direction, with new bases added to the 3'OH of the nascent polynucleotide. The 2003 discovery of a *Saccharomyces cerevisiae* tRNA^{His} guanylyltransferase (Thg1) demonstrated an exception to this rule and is at the center of this project (Gu et al., 2003). Thg1 possesses a set of intriguing features making it a paradigm among polymerase families – the first of its kind. *In vitro* and *in vivo* experiments clearly demonstrated that Thg1 has the ability to add a single non-Watson-Crick (non-WC) base paired guanosine (G_{-1}) to the 5' end of tRNA^{His} (Fig. 1) (Gu et al., 2003; Gu et al., 2005; Jackman & Phizicky, 2006a). Thus, the enzyme adds nucleotides in the 3'-5'direction – the reverse of all previously studied enzymes possessing polymerase capabilities. Thg1 catalysis consists of three steps: adenylylation which activates the tRNA, nucleotidyltransfer, and pyrophosphatase activity (Jahn & Pande, 1991; Gu et al., 2003) (Fig. 2).

*In vivo* in yeast, G_{-1} is incorporated across from a highly conserved adenosine (A_{73}). G_{-1} is a necessary recognition element for histidyl-tRNA synthetase, thus Thg1 is an essential gene (Fig. 1). G_{-1} is highly conserved across nearly all species from all three domains of life; only one other sequenced tRNA, tRNA^{Phe}, possesses any nucleotide at the -1 position (Schnare et al.,...
1985; Sprinzl et al., 1998; Wang et al., 2007). Previous work showed that G-1 is genomically encoded in prokaryotes and forms a Watson-Crick (WC) pair with C_73 while in eukaryotes G-1 is added post-transcriptionally and forms a non-WC pair with A_73 (Cooley et al., 1982; Orellana et al., 1986). Organisms possessing a genomically encoded G-1 result in RNase P cleavage that takes place one nucleotide upstream from the usual G_{+1} position of the RNase P reaction. Tying the two findings together, Thg1 was shown to be a highly conserved gene in species where G-1 is not encoded, thus which require Thg1 to add the G-1 recognition element (Gu et al., 2003; Abad et al., 2010).

Surprisingly, yeast Thg1 was shown to catalyze a second biochemical activity in vitro. In vitro reactions with a C_73 tRNA^{His} variant showed that Thg1 is capable of polymerizing multiple nucleotides in a WC fashion, albeit exclusively with C and G pairings (Jackman & Phizicky, 2006b). In doing so, it “travels” down the original template strand and replicates the substrate in the reverse direction (3’- 5’) until it runs into an A in the “template”. Although the mechanism is not known, A_73 appears to be of critical importance in deterring Thg1 from incorporating nucleotides past the -1 position. The two distinct enzymatic activities – WC versus non-WC – pose numerous mechanistic and evolutionary questions about tRNA^{His} and Thg1 catalysis, which can be answered by gaining a mechanistic understanding of Thg1 reactions.

The abilities to catalyze non-WC G-1 addition and polymerize WC base pairs are not restricted to tRNA^{His} and are likely to be a fundamental feature of the enzyme. In vitro Thg1 reaction with tRNA^{Phe} yielded a single incorporation of G-1 across A_{73} and a multiple base WC polymerization with a C_{73} tRNA^{Phe} variant (Jackman & Phizicky, 2006b). Indeed, the enzyme seems to follow a novel polymerase mechanism where A_{73} plays a critical role in controlling or perhaps modifying the reaction, ultimately guiding Thg1 to incorporate a single G residue. Aside
from the fact that this reaction is an exception to the exclusively observed 5’-3’ polymerase activity, it is of great interest to understand the two fundamentally distinct WC and non-WC activities exhibited by the enzyme. This surprising observation raises numerous questions as to how Thg1 interacts with the tRNA substrate and the incoming nucleotide substrates.

To investigate WC vs. non-WC addition we chose to focus on the guanylyltransfer step, hypothesizing that this step would be the most sensitive to the identity of the nucleotide incorporated by the enzyme. The initial step which activates the tRNA substrate is unlikely to be changed by the presence of a different nucleotide triphosphate (NTP). Also, the nucleotidyltransfer step can be carried out in either of the two biologically distinct processes: templated or non-templated addition. Therefore, we hypothesized that the identities of the substrate and the nucleotide may play a key role in determining the kinetic parameters for the nucleotidyltransfer step for WC vs. non-WC addition, and ultimately in determining the type of reaction that takes place.

By studying the rates of the nucleotidyltransfer step with different tRNA^{\text{His}} variants (A_{73}, C_{73} and G_{73}) with each of the 4 NTPs, we kinetically characterized the Thg1 nucleotidyltransfer step and found strong evidence for the existence of two modes of Thg1 catalysis. Thg1 follows a reaction of G_{1} addition where the selection of the non-WC paired G is not guided by preferential binding affinity for GTP (K_{D}). In contrast, with other N_{73} discriminators, similar kinetics are observed to those of canonical replicative polymerases where catalytic efficiency for correct WC incorporation is highly dependent on K_{D} for the incoming NTP; the enzyme shows an overwhelming (10^{5}–fold) preference for the complementary WC nucleotide (e.g. G_{1} across C_{73}) over a non-WC pairing NTP. Based on these results we hypothesize that A_{73} may act as a switch
to inhibit what may be an ancient 3'-5' polymerization activity of Thg1 resulting in the novel \textit{in vivo} non-templated G1 addition reaction used by eukaryotes.
RESULTS

Preparation and Purification of Human Thi1 (hThi1) Mutants. Previously performed alanine scanning of highly conserved residues in conjunction with a high resolution yThi1 crystal structure allowed us to hypothesize about the roles of various residues in Thi1 catalysis, such as ones critical for adenylylation (Jackman & Phizicky, 2008; Hyde et al., 2010). The yeast Thi1 (yThi1) and human Thi1 (hThi1) sequences share ~50% overall sequence similarity, therefore it was possible to identify homologous residues between yThi1 and hThi1. Based on previous work with yThi1 (Smith and Jackman, unpublished data), we hypothesized hThi1 residues K44 and N158 to play critical roles in the adenylylation step of G1 addition reaction (Fig. 3). Using QuikChange (Stratagene) we successfully created K44A, K44R, and N158D mutations in plasmids encoding hThi1. The mutant plasmids were confirmed by DNA sequencing. The three confirmed variant proteins were over-expressed in E. coli BL-21 DE3 pLysS strain and mutant hThi1 proteins were isolated by His6 tag affinity purification. The purity of mutant proteins was shown to be >95% by SDS-PAGE analysis (not shown).

K44 and N158 are Involved in Adenylylation in Human Thi1 (hThi1). Rates of adenylylation by each of the hThi1 variants was measured using a tRNAHis substrate that had been radiolabeled at the 5' end with 32P. The rates of adenylylation by the three single residue mutants (K44A, K44R, N158D) were measured under single turnover kinetic conditions. That is, by having a large excess of enzyme over the tRNA substrate in solution, we were able to measure the kinetics of just one step of catalysis by having all of the substrate consumed before a second round of catalysis can take place. The reactions were quenched with EDTA and digested with RNase A, followed by a CIP digestion (Fig. 4). Product protected from digestion by adenylylation (Ap*pGpC) and phosphate (*p) cleaved from unreacted substrate were resolved by
Thin Layer Chromatography (TLC). The quantified product conversion was fit to a single exponential equation:

\[ y = (Amp) \left( 1 - e^{-\left( k_{\text{obs}} \right)(t)} \right) \]  

(Equation 1)

where

\[ y \equiv \text{fraction of product conversion} \]

\[ Amp \equiv \text{amplitude of the curve} \Rightarrow \text{maximal product conversion} \]

Subsequently, the \( k_{\text{obs}} \) obtained for every concentration are plotted against the concentration of nucleotide and fit to:

\[ k_{\text{obs}} = (k_{\text{max}}) \frac{[NTP]}{K_D + [NTP]} \]  

(Equation 2)

The fit to these data yields \( k_{\text{max}} \), the maximal rate of the reaction being studied. Thus for the rate of adenylylation (activation step) \( k_{\text{max}} = k_{\text{aden}} \), and for the rate of the nucleotidyltransfer step \( k_{\text{max}} = k_{\text{ntrans}} \). Also, the \( K_D, NTP \) is a kinetic measurement of the thermodynamic \( K_D \) because rapid equilibrium kinetics have been observed for these reactions (Smith and Jackman unpublished).

Mutations of both residues, K44 and N158, had a modest effect on hThg1 catalytic efficiency of adenylylation (Table 1). \( k_{\text{aden}} \) measured with the K44A mutant was 0.118 ± 0.009 min\(^{-1}\) and 0.073 ± 0.006 min\(^{-1}\) for N158D. Therefore, compared to wild type, K44A and N158D resulted in a 5 and 8-fold decrease in the rate of adenylylation (\( k_{\text{aden}} \)), respectively. However, while K44A resulted in a significant loss of binding affinity for ATP with a \( K_D \) of 1300 ± 180 µM, N158D did not result in any significant changes having a \( K_D \) of 200 ± 80 µM. Compared to wild type \( K_{D, ATP} \) of 210 ± 20 µM, K44A compromised the binding affinity approximately 6-fold, while N158D retained an essentially identical \( K_D \) as the wt species. Overall, we report that changes in the rates of the reaction, combined with, in the case of K44, decreased binding affinity lead to a modest (5-10 fold) decrease on overall catalytic efficiency of the adenylylation...
step of catalysis for these hThg1 variants. These data are in contrast to the striking decreases in both rate of adenylylation and binding affinity for ATP for adenylylation observed for alterations at these same positions in yThg1.

It is interesting to note that, even a conservative mutation of lysine (K44R) did not retain the binding affinity for ATP observed with wt hThg1. In fact, the $K_D$ observed in K44R was nearly identical to one reported for K44A, where the side chain of residue 44 has been essentially removed. In contrast, while K44A resulted in a 5-fold decrease in the rate, K44R slightly sped up the chemistry with a 1.4-fold increase to $0.831 \pm 0.009 \text{ min}^{-1}$ rate of adenylylation. Given the results obtained with N158 (Table 1), it is reasonable to hypothesize that the residue is likely to be involved exclusively in the chemistry step of the reaction; however, due to absence of measurements of the same rates with an N158A mutant, we cannot affirm the aforementioned hypothesis. Overall, K44 mutations were characterized by an increase in $K_D$ while N158D resulted in a decreased $k_{\text{aden}}$ while preserving binding affinity (Table 1).

**Nucleotidyltransfer Step Across A_{73} is Affected by K44 But Not N158.** With yThg1, the effects of alteration of K44 or N161 (the analogous residue to N158) to alanine are exclusively limited to effects on the first (adenylylation) step of the G_{-1} addition reaction, and no effects on rate or $K_{D,\text{GTP}}$ for the second (nucleotidyltransfer) step are observed. However, in light of the differences described above with analogous hThg1 variant on adenylylation, we decided to also investigate the roles played by K44 and N158 residues in the nucleotidyltransfer step of the Thg1 reaction. Rates for this step, in which the incoming NTP is added to the activated tRNA, were measured for all three mutants. The substrate for this reaction was a ppp-tRNA[^His]$_{\text{32P}}$, labeled at the $\gamma$-phosphate (generated by *in vitro* transcription in presence of $\gamma$-[^32P]GTP). Kinetically, it has
been shown that ppp-tRNA$^{\text{His}}$ mimics the adenylylated-tRNA (Smith and Jackman, unpublished), with the major difference being in identity of leaving group following nucleotide addition (PPi for ppp-tRNA vs. AMP for ApptRNA). Since ppp-tRNA is readily generated by \textit{in vitro} reaction, we elected to use the triphosphorylated substrate throughout the entire study.

The nucleotidyltransfer assay (Fig. 5) was performed using *ppptRNA and hThg1 in presence of GTP, which will attack the activated tRNA generating G$_{\text{4,1}}$-containing product. As before, the reactions were quenched with EDTA and digested with RNase A. TLC was used to resolve the radiolabeled pyrophosphate which corresponded to products (*PPi) and unreacted *pppG$_{\text{4,1}}$pC which represents the remaining substrate following the RNase A digestion (Fig. 5). As before, the observed rates ($k_{\text{obs}}$) for each concentration of nucleotide (NTP) were fit to Equation 1 and subsequently a maximal catalytic rate and $K_{D,NTP}$ was obtained by fitting the plot of $k_{\text{obs}}$ vs. concentration of NTP to Equation 2.

The rate of G$_{\text{4,1}}$ incorporation into A$_{73}$-tRNA$^{\text{His}}$, $k_{\text{ntrans}}$, was measured under single turnover conditions described above. The rates measured for K44A (0.083 ± 0.007 min$^{-1}$) and N158D (0.078 ± 0.005 min$^{-1}$) did not significantly deviate from that measured previously for the wild type species (hThg1) (0.097 ± 0.005 min$^{-1}$), similar to what was observed previously for alterations of these residues in yThg1. However, the K44R alteration, initially hypothesized to be a key player in adenylylation, had an unexpected impact on the kinetics of nucleotidyltransfer. The mutation enhanced the rate of nucleotidyltransfer ($k_{\text{ntrans}}$) in excess of 4-fold to a rate of 0.43 ± 0.07 min$^{-1}$ (Table 2 and Fig. 6). While N158D was a factor exclusively in adenylylation, K44R had an impact on both reactions, particularly nucleotidyltransfer. Therefore, one interpretation of these results is that the kinetics of the enzyme are such that the rate of the chemistry is sacrificed
for an enhancement in the binding affinity. The implications of such selections are addressed further in Discussion.

Ribonucleotide at the 73rd Position of the Substrate tRNA Plays a Critical Role in Determining the Mode of Nucleotide Addition by S. cerevisiae Thg1. The reaction that initially caught the attention of the science community is the 3’-5’ addition of G₁ across A₇₃ catalyzed by the yeast Saccharomyces cerevisiae Thg1 (yThg1). However, yThg1 is also capable of catalyzing the templated 3’-5’ polymerization of nucleotides with variant tRNA^{His} substrates. The abundance of previous experimental data reported for the yeast homolog, including transient and steady state kinetics, made yThg1 the enzyme of choice to investigate differences between templated and non-templated 3’-5’ addition of nucleotides.

We explored a matrix of reactions to gain an insight to the mechanism of incorporating a non-WC base in the reverse direction of all other canonical polymerases yet retaining the capability of a WC 3’-5’ polymerization. Comparing the incorporation of each of the four NTPs across an A₇₃ versus C₇₃ and G₇₃ allowed us to learn about the kinetics governing each reaction. Until this study, it was known that yThg1 prefers to add non-WC G₁ to its physiological substrate (A₇₃-tRNA^{His}), whereas the NTP preference for N₁ addition to C₇₃ and G₇₃ tRNA^{His} was not known. Of all steps of Thg1 catalysis, we expected the nucleotidyltransfer step to be the most sensitive to the identity of the interaction (i.e. N₁:N₇₃), therefore we chose to study this step.

Using single turnover kinetics we measured the rates of nucleotide addition (k_{trans}) at the -1 position (N₁) testing the addition of each nucleotide (G₁, A₁, U₁, C₁) to A₇₃-tRNA^{His} and its variants. The most logical starting point was to compare the rates of incorporation across A₇₃. Interestingly, addition of WC base pairing U₁ was measured to take place at a rate of 0.77 ± 0.07
min\(^{-1}\), only a 4-fold decrease from G\(_{1}\) addition by \(\gamma\)Thg1 (2.84 ± 0.08 min\(^{-1}\), Smith and Jackman, unpublished). Conversely, A\(_{1}\) and C\(_{1}\) rate of non-WC addition occurred with drastically lower rates (0.032 ± 0.001 min\(^{-1}\) and 0.009 ± 0.001 min\(^{-1}\), respectively). Conversely, the \(K_D\) across all four nucleotides was practically unaffected by the identity of the NTP to be added to the tRNA (Table 3). Although there was a slight preference for GTP in the addition reaction with a slightly lower \(K_D\) of 23 ± 2 µM, the affinity for the other three nucleotides is quite comparable: 70 ± 15 µM for ATP, 140 ± 50 µM for UTP, and 200 ± 130 µM for CTP. Therefore, a difference in binding affinities for GTP versus the other three nucleotides is not likely to be a significant factor in the observed preference of \(\gamma\)Thg1 for GTP over other NTPs for addition to A\(_{73}\) tRNA\(^{\text{His}}\). The implications and novelty of this finding are addressed further in Discussion.

To investigate the substrate for templated G\(_{1}\) addition, a C\(_{73}\)-containing tRNA\(^{\text{His}}\) variant (i.e. C\(_{73}\)) was subjected to the same kinetic experiment under single turnover conditions. As before, the rate of incorporation at the -1 position was measured for all four nucleotides. The results were extremely different from that of A\(_{73}\). The enzyme showed an overwhelming preference for GTP for this reaction. In fact, the reactions were so fast that single exponential rates could not be accurately measured by manual quenching of reactions. Based on the plots of observed product formation (not shown) we estimate \(k_{\text{trans}}\) to be between 10 – 20 min\(^{-1}\) with a \(K_D\) ≤ 1 µM (Table 3). Our estimate was further supported by the same assay at pH 6.0. Previous unpublished data showed that \(k_{\text{trans}}\) drops approximately 12-fold at pH 6, opposed to pH 7.5, where all of the assays in this study were performed. Although it was similarly still not possible to accurately measure the rate of G\(_{1}\):C\(_{73}\), our data indicates an approximate rate of 1.2 min\(^{-1}\) at pH 6.0, approximately a 12-fold decrease from the rate at pH 7.5.
In contrast to G:1:C\textsubscript{73} facilitating the fastest rate, the highest binding affinity, and ultimately the highest catalytic efficiency reported thus far, incorporations of the non-WC pairing U, A, and C across from C\textsubscript{73} were measured to have a similarly low $k_{\text{trans}}$. The measured $k_{\text{trans}}$ for incorporation across C\textsubscript{73} were as follows: 0.14 ± 0.02 min\textsuperscript{-1} for U, 1.0 ± 0.01 min\textsuperscript{-1} for C, and 0.065 ± 0.005 min\textsuperscript{-1} for A. All three were over 100 times less than what was observed with GTP. In addition, Thg1 has a drastically weaker binding affinity for non-WC NTPs when compared to the sub-micromolar $K_D$ observed for GTP with C\textsubscript{73} tRNA\textsuperscript{His}. The next strongest affinity is 160 ± 40 µM (A:1:C\textsubscript{73}), a decrease in excess of 160 fold, followed by 510 ± 160 µM (U:1:C\textsubscript{73}), and 3.0 ± 0.5 mM (C:1:C\textsubscript{73}). To determine whether the strong preference for NTP was specific for GTP only, or could be observed with other WC pairing NTPs, we measured the rate of C\textsubscript{1} addition to G\textsubscript{73} (C:1:G\textsubscript{73}). Following the previously observed trend, Thg1 shows a significantly increased rate and enhanced affinity for CTP using a substrate that allows for WC addition (G\textsubscript{73}-tRNA) over the rate and $K_D$ observed for non-WC addition of CTP to A\textsubscript{73}-tRNA. The measured $k_{\text{trans}}$ and $K_D$ were 1.7 ± 0.2 min\textsuperscript{-1} and 7 µM, respectively; a 200-fold improvement in $k_{\text{trans}}$ and a 30-fold increase in $K_D,\text{NTP}$ over the corresponding rates for non-WC addition. Interestingly, the maximal rate of G\textsubscript{1} addition to C\textsubscript{73}-tRNA is yet even faster and occurs with a stronger binding affinity then the C:1:G\textsubscript{73} reaction, suggesting that yThg1 may also have an inherent preference for GTP (see Discussion).

We note that all of the measured $k_{\text{trans}}$ were derived from $k_{\text{obs}}$ which was obtained by fitting data to a single exponential curve (Equation 1). Indeed, the data recorded for the addition of each of the four nucleotides to A\textsubscript{73}-tRNA and for the non-WC additions to C\textsubscript{73}-tRNA are described well by the single exponential fit (not shown). Conversely, plots obtained for the WC base pairing combinations of NTP and N\textsubscript{73}-tRNA (C:1:G\textsubscript{73} and G:1:C\textsubscript{73}) were not as well
described by the single exponential equation. When WC paired NTPs were added to a non A\textsubscript{73}-containing tRNA, the data were better described by the double exponential equation; particularly at high concentrations of NTPs:

\[ y = (Amp_1)(1 - e^{-(k_{obs1})(t)}) + (Amp_2)(1 - e^{-(k_{obs2})(t)}) \]  

(Equation 3)

\[ y \equiv \% \text{ product conversion} \]
\[ Amp \equiv \text{amplitude of the curve} \Rightarrow \text{maximal product conversion} \]

This phenomenon observed for WC addition across C\textsubscript{73} and G\textsubscript{73}, was not observed for U\textsubscript{1} during addition across A\textsubscript{73} (not shown). The evidence for a double exponential behavior implies the presence of two inherent rates for the reactions of interest – a slow and a fast rate exhibited by the enzyme. While the double exponential behavior is clearly supported by the plots (Fig. 7), at this time we cannot accurately report the fast rate because of reaction speeds exceeding the capabilities of gathering timepoints by manual quench; however, we are able to present reasonable estimates because the single exponential fit provides a lower limit to the actual rate of the \( k_{\text{trans}} \). Nonetheless, the complexity of the kinetic data obtained for the WC additions to N\text{73}-tRNAs other than A\textsubscript{73}-tRNA\textsuperscript{His} is additional evidence that different modes of catalysis are occurring with the two types of tRNA substrates.

\textit{BtThg1 is Capable of Incorporating 5' Guanosine Analogues at +1 position.} One potential use for Thg1 activity is to create ways to 5' label nucleic acids, a technology that is currently not available. Also, development of such technologies has potential to be useful in study of the mechanism of specificity for various NTPs. To investigate these capabilities we performed activity assays on incorporation of several GTP analogues (\textbullet\textsuperscript{G}) of interest at the +1 position on tRNA\textsuperscript{His} (Fig. 8). The substrate was prepared in a similar fashion as the one used for adenylylation assays, except G\textsubscript{+2} was 5'-radiolabeled with \textsuperscript{32}P (see description above and
Methods section). Previous data showed that *Bacillus thuringiensis* Thg1 (BtThg1) exhibits more flexibility with respect to NTP and tRNA substrates that can be accommodated in the 3’-5’ addition reaction (Rao et al., 2010). Therefore, BtThg1 was the enzyme of choice for this set of experiments. The studied 5’ analogues were all such that the triphosphate group was replaced by a moiety of interest including an azido group, a biotin molecule, and the Alexa555 dye. All three analogues were prepared by CLICK chemistry (provided by Subha Das, Carnegie Mellon University) to a high yield and purity. Initial results showed that BtThg1 was active with all three analogues. However, at that point the identity of the TLC spots (not shown) was not clear. The two possibilities were that the reaction stopped after activating the tRNA (Ap*pG+2pC…) or progressed to successfully incorporate the guanosine analogue (▪G+1*pG+2pC). To further prove the identity of the spots on the TLC plates, the products of RNase A and CIP digestion were treated with RNase T1. The enzyme cleaves 3’ to G thus yielding either an Ap*pG+2pC or a *pG+2 which can readily be resolved by two TLC systems described in Methods. Digestion with RNase T1 did in fact prove the identity of the products to be an approximately 1:1 mix of both of the possible products. Therefore, BtThg1 is capable of incorporating the analogues, albeit to a lower degree of efficiency when compared to +1 incorporation of unmodified GTP. We note here that the experiments carried out with the bright pink Alexa555 dye were also analyzed with fluorescence. The results obtained by fluorescence were in agreement with the results obtained from the same TLC plate by the traditional means of radiolabeling. Finally, while we were able to show that BtThg1 is capable of incorporating useful analogues of guanosine, the kinetics of the reactions were not measured at this time but will be considered in future studies.
DISCUSSION

*In vivo* in yeast Thg1 catalyzes the addition of a highly conserved G₁ to tRNA^{His}. The novelty of Thg1 is the fact that the enzyme is capable of adding residues in a 3'-5' direction to a tRNA substrate. The *in vivo* G₁ addition reaction is a non-WC dependent reaction where a GTP is added across A₇₃. Previous work showed the enzyme to possess a capability of performing a second biochemical activity – polymerization of WC base pairing nucleotides – albeit only with G:C pairing. In this study we were able to further explore the mechanism of the various reactions catalyzed by Thg1 family enzymes using single turnover kinetic techniques. First, using the newly acquired hThg1 crystal structure we tested at least two residues for their roles in the activation step for the 3'-5' addition reaction. Second, we carried out an extensive kinetic study on a matrix of nucleotidyltransfer reactions catalyzed by yThg1 at the -1 position. Through this study we present strong evidence for two separate mechanisms used by Thg1 to select incoming NTPs for the nucleotidyltransfer reaction. We showed that the identity of the nucleotide at position 73 determines whether selection of incoming NTP occurs primarily through activation of chemistry such as for non-WC addition to A₇₃, or whether a preference for binding correct WC pairing NTP contributes significantly to catalysis as observed for other N₇₃ tRNAs. In other words, we believe that preferential binding of the incoming NTP does not play a role for non-WC addition (facilitated by the presence of A₇₃), while it is a factor for WC reactions across other nucleotides. Also, based on the data from this study it is likely that Thg1 has an inherent preference for GTP while A₇₃ is a strong determinant for the mode of Thg1 catalysis. Finally, we showed that BtThg is capable of successfully incorporating at least three useful 5' GTP analogues into the +1 position of tRNA^{His}, a finding with implications for future work in the lab.
To expand our understanding of Thg1 and utilize the newly available crystal structure of hThg1, we tested key adenylylation residues previously identified in yThg1 in the context of the human homolog. It is interesting to note that despite significant effects on $k_{\text{aden}}$ with the same alteration in yeast, the K44A mutation resulted in only a small reduction in the rate of adenylylation, where the drop was only 5-fold. The results show that while K44 may be involved in adenylylation, retention of some adenylylation activity suggests that K44 is not the primary residue involved in the chemistry of the reaction. Increased $K_D$ suggests that K44 is highly involved in binding the nucleotide, similar to what is observed with the yeast enzyme. Decreased $k_{\text{aden}}$ seems to be manifested in the mutation to alanine which resulted in loss of a critical interaction between the NTP and the enzyme, thus slowing the rate of the reaction. In contrast, mutating the same residue to arginine, an alteration that retains the positive charge of the wild-type lysine residue, resulted in an essentially identical loss of binding affinity to the alanine alteration, yet a slightly increased $k_{\text{aden}}$. One interpretation of this result may be that the more bulky arginine is incapable of facilitating effective binding due to steric hindrance. Furthermore, we note here that according to the currently available crystal structure (Hyde et al., 2010) which shows a dGTP bound in the active site of a yThg1 dimer, the part of the nucleotide that is closest to K44 is the guanosine base. These distances between K44 and the bound NTP vary between 5-10 Angstroms – too far to react at the given orientation (Fig. 9). Also, no other amino acid residues are in proximity to K44 to be activated by the amine.

Several possible scenarios may explain the observed phenomenon. K44 may interact with the tRNA rather than the nucleotide. It is logical to assume that the tRNA binds in close proximity to the nucleotide and the lysine residue is close enough to assist in binding the tRNA while far enough not to interfere with the nucleotide. FRET experiments or a crystal structure
with a bound tRNA would help to address this hypothesis. Another possible scenario is that the enzyme undergoes a conformational change which brings the nucleotide and K44 in proximity. The hypothesis is in accord with our kinetic studies (addressed in more detail later) where double exponential character suggests that the enzyme may have two or more conformations resulting in the slow and fast rates (e.g. K44 in the current distal conformation may give the slow rate which could be enhanced when K44 is brought in proximity by a conformational change). Finally, it is possible that the reaction is a combination of both. The available crystal structure shows a bound dGTP, thus the crystal structure does not reveal the presence of a 2’ OH present in all ribonucleotides, such as the ATP used for the activation step. According to PYMOL, the 2’ OH should be approximately 1.4 Å. Therefore, in reality the amine of lysine has another hydroxyl that it could possibly interact with. With that in mind, we further develop this hypothesis: K44A results in a neutral residue that simply doesn’t reach the nucleotide to interact with the partially negative oxygen. Furthermore, K44R may result in a similar loss of binding affinity for two compounding reasons. First, the positive charge normally available in lysine is spread over 3 atoms weakening the interaction with the partially negative oxygen. Second, the arginine side chain is 1 atom longer and may now result in weakened attraction between the 2’ OH and the residue.

The last hypothesis is supported by the kinetics that we report for the succeeding nucleotidyltransfer step of the reaction. K44A resulted in $k_{\text{aden}}$ and $K_D$ that were comparable to the wild type species. Conversely, K44R resulted in a slightly higher $K_D$ and a 4-fold increase in $k_{\text{trans}}$. Since positive charge is spread out over three amines, electrons on these amines are available for interaction. Combined with the assumption that arginine is now capable of reaching the 3’ OH, the side chain may form partial interactions with the hydrogen helping to activate the
3' OH for a nucleophilic attack. (Previous published and unpublished data strongly suggests that although the enzyme does not share sequence homology, its active site strongly resembles ones of canonical polymerases (*e.g.* Pol I) and employs a 2 metal ion catalysis mechanism that also acts to activate the 3' OH.) An experiment of interest to prove or disprove the suggested hypothesis is to create a K44E where the negative charge would repel the partial negative of the oxygen while being too far to interact with the 3' OH hydrogen resulting in a low binding affinity and $k_{\text{trans}}$ comparable to one observed in the wt species. In conclusion to this experiment, regardless of the actual scenario, it appears that an evolutionary choice was made where the rate of chemistry was compromised for a stronger binding affinity.

In contrast to K44, our data supports the hypothesis that N158 is likely to be involved primarily in the chemistry of the adenylylation step of Thg1 catalysis. This conclusion is supported by an unaffected $K_D$ in both of the studied steps of the reaction and an unaffected $k_{\text{trans}}$ while $k_{\text{aden}}$ experienced a significant decrease. In accordance with this hypothesis, the N158A homolog in yThg1 resulted in loss of adenylylation activity in excess of 500 fold. Using kinetics, the residue was demonstrated to be involved in the catalysis yet the crystal structure shows the residue to be around 9 Å away from the closest point of contact with the bound nucleotid. This is in accord with the previously suggested hypothesis that the enzyme is likely to undergo a conformational change. A future experiment of interest is to successfully create an N158A mutant and subject the protein to a similar set of kinetic experiments. It is likely that N158A will cause $k_{\text{aden}}$ to experience an even bigger drop – confirming our hypothesis about the role of the residue.

To better understand the difference of what drives Thg1 to incorporate a non-WC $G_{j:1}:A_{73}$ in certain cases while performing a WC polymerization under a different set of conditions, we
performed a study measuring kinetics of the nucleotidyltransfer step using tRNAs with various
N$_{73}$ nucleotides (see Results for reasons to study this particular step of catalysis). Our studies
highlighted several key points outlining the rules of Thg1 reaction which will serve to help us
understand the mechanism employed by Thg1. When designing an enzyme specific for a certain
substrate, binding affinity is a substantial contribution and is often observed to be a strategy to
discriminate between correct and incorrect substrates. The strategy is particularly popular among
canonical replicative polymerases, which exhibit much stronger binding affinity for an incoming
NTP that is capable of WC base pair formation than for an "incorrect" NTP that can not make a
WC base pair with the template nucleotide.

Thg1 displays yet another exception to a general rule even at this level. Indeed, the
observed $K_D$ for G$_{-1}$ addition is $23 \pm 2 \mu M$ while the highest $K_D$ reported among the four
nucleotides is only $200 \pm 130 \mu M$ (C$_{-1}$:A$_{73}$). A discrepancy of 10-fold by itself is not enough to
ensure the fidelity of the reaction. Canonical polymerases often favor WC over non-WC
nucleotides by differences in $K_D$ in excess of 100-fold and more. The finding clearly shows that
binding affinity of nucleotides during addition across A$_{73}$, at best, has a mild effect. Furthermore,
the enzyme seems to sacrifice the rate of nucleotidyltransfer chemistry ($k_{ntrans}$) for $K_{D,ATP}$ in $k_{aden}$.
In addition to the aberrant rules of guanosine selection, the enzyme showed an appreciable rate
of U$_{-1}$ incorporation. At this point Thg1 addition of U$_{-1}$ to an A$_{73}$ is the only known instance in
our studies where the WC pairing happens at a rate that is slower than that of a non-WC
nucleotidyltransfer. This observation suggests that while the enzyme has an inherent, perhaps
ancient, ability to perform a 3'->5' polymerization, A$_{73}$ acts as a switch that causes the chemistry
of the reaction to favor non-WC addition (i.e. G$_{-1}$:A$_{73}$). In addition, previous findings show that
an adenosine acts as a terminator of multiple turnover polymerization (Jackman & Phizicky, 2006b).

Initially, we set out to find what drives Thg1 to behave the way it does. For reasons above, we hypothesize that presence or absence of A_{73} is at least one critical determinant of Thg1 activity. Overall, we found that an enzyme that serves to add a vital discriminant to tRNA^{His} shows a comparable K_{D} for all four nucleotides and a catalytic efficiency of just 17 fold less for incorporation of an "incorrect" (non-G) nucleotide. This finding, of course, indicates that the enzyme employs additional strategies in ensuring fidelity. Alternatively, there may be an undiscovered Thg1 proofreading activity. Finally, both hypotheses may be true.

In contrast, addition across from other discriminator nucleotides was highlighted by features found in canonical replicative polymerases. Thg1 shows an overwhelming preference for NTPs that are capable of forming WC pairs with binding affinities that exceed affinities for non-WC pairing nucleotides by at least 100-fold – a characteristic of canonical polymerases. We also report that WC addition across from nucleotides other than adenosine seems to be distinct on a fundamental level. While the currently hypothesized in vivo, physiological Thg1 reaction of G_{1}:A_{73} addition in eukaryotes occurs at a rate of 2.84 ± 0.08 min^{-1}, G_{1}:C_{73} was greater than 10 min^{-1}. The results beg the question of why does the enzyme possess an “unused” activity with a catalytic efficiency that is over 100 times greater than the physiological Thg1 reaction. Possible explanations include that Thg1 and current canonical polymerases share a common ancestor where evolution made a choice of 5'-3' replication while Thg1 evolved to perform the known G_{1} addition. In addition, Thg1 may use its WC 3'-5' polymerization in other physiological processes that are yet to be discovered. The previously thought rule of 5'-3' addition results in disadvantages to the organism and it would be irrational for a biological system to evolve where
it puts itself at a disadvantage. An example of such an instance is the perpetual loss of genomic information from primer binding sites during DNA replication. Thg1 possesses the capability to incorporate DNA (Jackman and Phizicky, unpublished data). Thg1 may be among several enzymes that are yet to be discovered that counterbalances the disadvantages caused by the exclusivity of genomic replication.

Our data also supports the idea that A$_{73}$ seems to act as a molecular switch causing distinct Thg1 activity that differs from activities observed across with other N$_{73}$ nucleotides. It is reasonable to hypothesize that the presence of the adenosine at position 73 of the substrate tRNA causes or prevents a certain shift of the active site or interaction effectively acting as a determinant for the type of the reaction. At this time we believe A$_{73}$ to act in the former of the two proposed scenarios. All additions across A$_{73}$ and all non-WC additions were described well by a single exponential fit (Equation 1). Conversely, WC additions across C$_{73}$ and G$_{73}$ were fit significantly better by a double exponential curve (Equation 3), particularly at high concentrations of NTP. In other words, the kinetics of the reaction approached single exponential character as the reaction was slowed due to dependence on nucleotide concentration. The demonstrated existence of double exponential behavior which dominates exclusively for WC addition across the studied A$_{73}$ variants strongly suggests that such scenarios are described by two rates – a slow and a fast rate. Building on the observation, it is likely that the enzyme evolved such that its default conformation is ideally adapted for binding a tRNA$^{His}$ possessing an A$_{73}$ and performing G$_{-1}$ addition, hence negating the need for strong discrimination by binding affinity.

For reasons above, it is likely that the enzyme evolved from a common ancestor of modern polymerases and may be capable of reverting to the ancient activity by adopting a
different conformation. Thg1 was previously reported to polymerize exclusively G:C additions. It would be fitting for a G or a C, or perhaps both to trigger an interaction that converts Thg1 into its canonical polymerase form. This canonical conformation is the conformation contributing to the double exponential behavior observed above. The presence of the fast rate dissipates when the reaction is limited by nucleotide concentration.

One possibility is that the enzyme must be switching between two states or conformations which give the slow and the fast rate. This hypothesis resonates with the conclusions above which we presented from the studies of hThg1 where we suggested that the enzyme is likely to undergo conformational changes during catalysis. If the hypotheses above are in fact the case, it may help to explain the transition from double exponential to a single exponential behavior as the rate of the nucleotidyltransfer reaction is limited by NTP concentration.

In addition to kinetic studies, to further expand our understanding of mechanisms employed by Thg1, we studied the incorporation of various GTP analogues at the +1 position. Although the project is still in an early stage we were able to learn that the triphosphate moiety of the nucleotide can be explored as a region of interest to create useful analogues. For example, various markers such as biotin or Alexa555 dye can be attached to the tRNA. Biotin can be used for pull down assays to study tRNA-protein interactions while Alexa555 can be used as an additional or an alternative to labeling with $^{32}$P for assays. Future work on this project will focus on incorporation of these and other analogues at the -1 position as well as further development of potential experimental tools. By comparing incorporation at the +1 position versus incorporation at the -1 position we hope to learn about the roles of triphosphate in what has already been shown to be a unique interaction between Thg1 and the 73$^{rd}$ position of tRNA$^{\text{His}}$. 
A full mechanistic understanding will serve as a paradigm in discovery, study, and useful modifications (i.e. biomedical or biotech applications) of the enzyme. The newly obtained kinetic understanding in conjunction with crystal structure work serve as a foundation for testing other Thg1 variants and homologs ultimately leading to a full mechanistic understanding of the dual modes of catalyses exhibited by the novel Thg1 polymerase.
METHODS

Expression and Purification of Human Thg1 Mutants. Mutant DNA sequences were created from a human wild type Thg1 vector (JEJ 419) with the QuikChange mutagenesis kit (Sigma) according to manufacturer instructions and confirmed by The Ohio State University Plant-Microbe Genomics Facility. Plasmids with correct sequences were transformed into E. coli BL-21 DE3 pLysS competent cells. His$_6$ tagged hThg1 mutants were over expressed in LB Amp$^+$ liquid media (grown at 37 °C, 5 hrs., induced with IPTG at 18 °C, O/N). Cells were lysed using a French-press and proteins were purified from the soluble crude extract by His$_6$ TALON affinity beads. Fractions of eluted sample were qualitatively assessed for protein content by visual inspection using Bio-Rad dye. Fractions of high protein content were pooled and dialyzed overnight in 1 L of 50 % glycerol buffer consisting of 20 mM HEPES (pH 7.5), 0.5 M NaCl, 4 mM MgCl$_2$, 1 µM EDTA, 1 mM DTT. SDS-PAGE analysis was used to confirm the purity and correct molecular weight (suggesting correct identity) of the protein. Wild type yThg1 was purified in a similar manner described above. Wild type plasmids were transformed into E. coli BL-21 DE3 pLysS. The resulting strains were over expressed in LB Amp$^+$ liquid media (grown at 37 °C, 5 hrs., induced with IPTG at 18 °C, O/N). Protein variants were purified per protocol above.

Preparation of tRNA$^{His}$ Radiolabeled Substrate *ptRNA$^{His}$ was obtained using an in vitro labeling protocol as described in Rao et. al. 2010. Briefly, in vitro T7 RNA Polymerase transcribed ppptRNA$^{His}$ was phosphatase treated followed by a kinase reaction using T4 Polynucleotide Kinase and $^{32}$P-$\gamma$-ATP. The labeled product (*ptRNA$^{His}$) was gel purified by 10% acrylamide 4 M urea PAGE, isolated by phenol:chloroform:isoamyl alcohol (25:24:1) extraction,
ethanol precipitated, and resuspended in TE pH 7.5 buffer. *ppptRNA\textsuperscript{His} was prepared by an \textit{in vitro} transcription using T7 RNA Polymerase in the presence of γ\textsuperscript{-32}P-GTP, gel-purified, and stored in a similar manner.

\textbf{Adenylylation Assay.} All adenylylation assays were performed with 5\textsuperscript{′}\textsuperscript{-32}P- tRNA\textsuperscript{His}. Each reaction contained 25 mM HEPES pH 7.5 buffer, 10 mM MgCl\textsubscript{2}, 3 mM DTT, 125 mM NaCl, 0.2 mg/ml BSA, 15 µM protein (previous assays determined 15 µM to be the saturating protein concentration for maximal rate), and \textsuperscript{32}P labeled tRNA\textsuperscript{His}. The reaction was initiated by adding the mix to a variable concentration of ATP. 5 µL reaction aliquots were quenched with a 1 µL mix of 250 mM EDTA pH 8 and 5 mg/mL RNase A. The aliquots were digested with RNase A (50 ºC, 10 min.), followed by a digestion with 1 unit of CIP (37 ºC, 35 min.) and resolved by silica gel TLC, in a 55:35:10 1-Propanol:NH\textsubscript{4}OH:H\textsubscript{2}O solvent system. The results were visualized with phosphor imaging and quantified with ImageQuant 5.0 software.

\textbf{Nucleotidyltransfer Assay.} All nucleotidyltransfer assays were performed with 5\textsuperscript{′} γ\textsuperscript{-32}P radiolabeled tRNA\textsuperscript{His} (*ppptRNA\textsuperscript{His}). Each reaction contained 25 mM HEPES pH 7.5 buffer, 10 mM MgCl\textsubscript{2}, 3 mM DTT, 125 mM NaCl, 0.2 mg/ml BSA, 15 µM protein, and \textsuperscript{32}P labeled tRNA\textsuperscript{His}. The reaction was initiated by adding the mix to a variable concentration of nucleotide that was to be added to the -1 position. 3 µL reaction aliquots were quenched with a 1 µL mix of 250 mM EDTA pH 8 and 5 mg/mL RNase A. The aliquots were digested with RNase A (50 ºC, 10 min.) and resolved by polyethylene imine cellulose TLC, in a 80:20 0.5 M KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4} pH 6.3: methanol solvent system. The results were visualized with phosphor imaging and quantified with ImageQuant 5.0 software.
**Preparation of Guanosine Analogues.** The analogues used in this experiment were received from Dr. Subha Das (Carnegie Mellon University) and were prepared by CLICK chemistry in the Das laboratory. All three analogues were received as a powder. The powder was resuspended in 1 M Tris pH 7.5 buffer and centrifuged. Decant was saved and concentration of the analogue species was determined using UV spectroscopy where $\varepsilon_{260}$ of GTP (11,750 L*mol$^{-1}$*cm$^{-1}$) was used for analogues. Stock concentrations of analogues were determined to be 14 mM for the azido analogue, 10 mM for the biotin analogue, and 10 mM for the Alexa555 analogue. Stocks were stored at -20 °C.
**Figure 1:** Thg1 catalyzes a novel reaction where $G_{-1}$ is incorporated across from a highly conserved adenosine ($A_{73}$). $G_{-1}$ is a necessary recognition element for aminoacyl-tRNA$^{\text{His}}$ synthetase and is therefore an essential gene in species that do not have a genomically encoded $G_{-1}$. 
Figure 2: Previous work identified the minimal kinetic mechanism above. Thg1 catalysis is described by at least three steps: 1 – adenylylation which activates the tRNA substrate; 2 – nucleotidyl transfer where the incoming nucleotide (i.e. GTP) is added in a 3’-5’ fashion; 3 – pyrophosphatase activity which releases a PPI.
Figure 3a: Wild type human Thg1 and wild type yeast Thg1 amino acid sequences alignment.

Note: *H. sapiens* homolog has a preceding 29-residue N-terminal mitochondrial targeting sequence domain omitted from this alignment.
Figure 3b: K44, N158, and bound dGTP of human Thg1(hThg1). Previous kinetic data suggest that the dGTP mimics the position of the ATP nucleotide that participates in the first, activation step of Thg1 catalysis.
Adenylylation Assay: Measuring the rate of adenylylation of tRNA$^{\text{His}}$ by Thg1

**Figure 4:** The assay used to measure the rate of adenylylation ($k_{\text{aden}}$) by Thg1.
<table>
<thead>
<tr>
<th>Thg1 Species</th>
<th>tRNA Substrate</th>
<th>$k_{\text{aden}}$ (min$^{-1}$)</th>
<th>$K_D$ (µM)</th>
<th>$k_{\text{aden}}/K_D$ (min$^{-1}$/µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>$A_{73}$</td>
<td>0.58 ± 0.02</td>
<td>210 ± 20</td>
<td>0.0028</td>
</tr>
<tr>
<td>K44A</td>
<td>$A_{73}$</td>
<td>0.118 ± 0.009</td>
<td>1300 ± 180</td>
<td>9.0E-5</td>
</tr>
<tr>
<td>K44R</td>
<td>$A_{73}$</td>
<td>0.831 ± 0.16</td>
<td>1300 ± 500</td>
<td>6.0E-4</td>
</tr>
<tr>
<td>N158D</td>
<td>$A_{73}$</td>
<td>0.073 ± 0.006</td>
<td>200 ± 80</td>
<td>4.0E-4</td>
</tr>
</tbody>
</table>

Table 1: Rate of adenylylation ($k_{\text{aden}}$) of tRNA$^{\text{His}}$ by human Thg1 (hThg1) were tested in three mutants showing that K44 and N158 play a role in the adenylylation reaction. The results suggest an evolutionary choice for a stronger binding affinity over the rate of the reaction.
**Nucleotidyltransfer Assay: Measuring the rate of Thg1 nucleotidyl transfer across A73 in tRNAHis**

**Figure 5:** Assay used to measure the rate of Thg1 nucleotidyl transfer reaction and a sample TLC PEI cellulose F plate used to resolve the reactants and products.
<table>
<thead>
<tr>
<th>Thg1 Species</th>
<th>tRNA Substrate</th>
<th>$k_{\text{trans}}$ (min$^{-1}$)</th>
<th>$K_D$ (µM)</th>
<th>($k_{\text{trans}}/K_D$) (min$^{-1}$/µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>$A_{73}$</td>
<td>0.097 ± 0.005</td>
<td>10 ± 5</td>
<td>1.0E-02</td>
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<tr>
<td>K44A</td>
<td>$A_{73}$</td>
<td>0.083 ± 0.007</td>
<td>40 ± 10</td>
<td>2.0E-03</td>
</tr>
<tr>
<td>K44R</td>
<td>$A_{73}$</td>
<td>0.43 ± 0.07</td>
<td>110 ± 40</td>
<td>4.0E-03</td>
</tr>
<tr>
<td>N158D</td>
<td>$A_{73}$</td>
<td>0.078 ± 0.005</td>
<td>12 ± 3</td>
<td>7.0E-03</td>
</tr>
</tbody>
</table>
Figure 6: $k_{n\text{trans}}$ was determined by fitting experimental data with Equation 1 and Equation 2. While K44 was shown to play a role in binding the substrate, the mutant also resulted in a 4-fold enhancement of $k_{n\text{trans}}$ over the wild type hThg1.
<table>
<thead>
<tr>
<th>NTP</th>
<th>tRNA Substrate</th>
<th>$k_{\text{intrans}}$ (min$^{-1}$)</th>
<th>$K_D$ (µM)</th>
<th>($k_{\text{intrans}}/K_D$) (min$^{-1}$/µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>A$_{73}$</td>
<td>2.84 ± 0.08</td>
<td>23 ± 2</td>
<td>1.2E-01</td>
</tr>
<tr>
<td>U</td>
<td>A$_{73}$</td>
<td>0.77 ± 0.07</td>
<td>140 ± 50</td>
<td>5.5E-03</td>
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<tr>
<td>A</td>
<td>A$_{73}$</td>
<td>0.032 ± 0.001</td>
<td>70 ± 15</td>
<td>4.6E-04</td>
</tr>
<tr>
<td>C</td>
<td>A$_{73}$</td>
<td>0.009 ± 0.001</td>
<td>200 ± 130</td>
<td>4.5E-05</td>
</tr>
<tr>
<td>*G</td>
<td>C$_{73}$</td>
<td>10 - 20</td>
<td>≤ 1</td>
<td>≥ 1.5E+1</td>
</tr>
<tr>
<td>U</td>
<td>C$_{73}$</td>
<td>0.14 ± 0.02</td>
<td>510 ± 160</td>
<td>2.7E-04</td>
</tr>
<tr>
<td>A</td>
<td>C$_{73}$</td>
<td>0.065 ± 0.005</td>
<td>160 ± 40</td>
<td>4.1E-04</td>
</tr>
<tr>
<td>C</td>
<td>C$_{73}$</td>
<td>0.10 ± 0.01</td>
<td>3000 ± 500</td>
<td>3.3E-05</td>
</tr>
<tr>
<td>*C</td>
<td>G$_{73}$</td>
<td>1.7 ± 0.2</td>
<td>7 ± 3</td>
<td>2.4E-1</td>
</tr>
</tbody>
</table>

Lower limit to $k_{\text{intrans}}$ derived from single exponential fits to determine $k_{\text{obs}}$ at high concentrations of NTP.
Figure 7: The data for WC addition across C\textsubscript{73} and G\textsubscript{73} were fit the best by a double exponential curve while U\textsubscript{1}:A\textsubscript{73} was described well by a single exponential fit.
Figure 8: Analogues put into G_{+1} by BrThg1. Useful analogues such as ones demonstrated above give mechanistic insight as well as show potential as future experimental tools.

- R
- N≡N⁺ =N⁻
- Biotin
- Alexa555 (dye)
Figure 9: Measurements of potential interactions between K44 and a bound ribonucleotide. The crystal structure above demonstrates a dGTP bound in hThg1.
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