The role of arginine 244 in *Candida glabrata* tRNA nucleotidyltransferase

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A Thesis

In The Department

of

Chemistry and Biochemistry

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science

(Chemistry) at Concordia University

Montreal, Quebec, Canada

April 2009

Your file Votre référence
ISBN: 978-0-494-63337-3
Our file Notre référence
ISBN: 978-0-494-63337-3

ABSTRACT

The role of arginine 244 in *Candida glabrata* tRNA nucleotidyltransferase

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The enzyme ATP(CTP):tRNA nucleotidyltransferase is essential in eukaryotes to allow tRNAs to participate in protein synthesis. It adds a specific cytidine-cytidine-adenosine (CCA) sequence to generate positions 74, 75, and 76 at the 3' ends of tRNAs in a nucleic acid template-independent manner. Considerable effort has gone into exploring the catalytic mechanism of this enzyme and several bacterial forms, as well as the human enzyme, have been crystallized either in the presence or absence of substrates. Here, we will discuss the role of an essential conserved arginine in a novel attempt to link *in vitro* enzyme activity to a measurable *in vivo* phenotype. We found that changing arginine 244 in *Candida glabrata* tRNA nucleotidyltransferase to alanine, lysine, or methionine resulted in cell death, even though the variant enzymes showed 50-70% activity as compared to the wild-type enzyme under standard *in vitro* assay conditions using a mixed population of tRNAs as substrate. More specific assays monitoring the incorporation of nucleotides into tRNA with known 3' ends demonstrated a loss of nucleotide addition at position 74, a reduction of nucleotide specificity at position 75, and an increase in nucleotide specificity at position 76 as compared to the native enzyme. These results suggest that arginine 244 in *C. glabrata* tRNA nucleotidyltransferase plays a role not only in binding and orienting the substrates (nucleotide triphosphates and tRNA) for catalysis but also in excluding ATP from the active site prior to nucleotide addition to positions 74 and 75.

Acknowledgements

I would like to thank Dr. Paul Joyce for allowing me to grow with this project. My thanks as well for not only allowing me to learn and take initiative as obstacles arose, but also for being a constant source of valuable advice and guidance when I could not move forth on my own. I am ever grateful for his dedication and time spent reading and editing this thesis.

I would like to thank my committee members Dr. Joanne Turnbull and Dr. Peter Pawelek for their guidance and counsel throughout the course of my research.

I would like to thank Dr. Pamela Hanic-Joyce for the pleasure of her support and companionship throughout these past few years. Thank you for making the lab an interesting place for work and dialogue.

I would like to thank Jamie who has always stood by my side throughout the many hardships of this experience as well as the successes. Thank you for your patience with regards to my constant absences and my long hours in the lab. Thank you for always being there.

I would like to thank my parents and my brothers whom have been a constant source of support and understanding.

I would like to thank Nadia for being a true friend and companion throughout this experience and for always being available to get me out of a bind.

Finally, I would like to thank all my friends and family who have waited patiently throughout my studies and who have always rooted for my success.

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List of Abbreviations

A.U.: Absorbance units
ALA: Alanine
Amp: Ampicillin
AMP: Adenosine monophosphate
APS: Ammonium persulfate
ARG: Arginine
ATP: Adenosine triphosphate
BSA: Bovine serum albumin
CD: Circular Dichroism
CTP: Cytidine triphosphate
dH_2O: Distilled water
DNA: Deoxyribonucleic acid
EDTA : Ethylene diamine tetra-acetic acid
EtBr: Ethidium bromide
FOA : 5-fluoro-orotic acid
GST: Glutathione-S-transferase
GTP: Guanosine triphosphate
H-bond: Hydrogen bond
HCl : Hydrochloric Acid
IPTG: Isopropyl-β-D-Thiogalactopyranoside
K_m: Michaelis-Menton constant
mdeg: Millidegrees
MET: Methionine
NTP: Nucleoside triphosphate
PBS : Phosphate-buffered saline
PCR : Polymerase chain reaction
PEG: Polyethylene glycol
RNA: Ribonucleic acid
rpm: Rotations per minute
SDS: Sodium dodecyl sulfate
SC: Synthetic complete
TBE: Tris-boric acid-EDTA
TdT: Terminal deoxynucleotidyltransferase
TEG: Tris-EDTA-glucose
TEMED: Tetramethylethylenediamine
Tm: Melting temperature
tRNA: Transfer ribonucleic acid
UTP: Uracil triphosphate
UV: Ultraviolet
YT: Yeast Tryptone

1. Introduction

1.1 Transfer RNA (tRNA)

Transfer RNA (tRNA) acts as 'an RNA intermediate' to direct the incorporation of amino acids into proteins (Holley *et al.*, 1965; Soll and RajBhandary, 1995). Transfer RNAs are easily recognizable by their unique clover-leaf secondary structures which consist of three conserved stem/loop regions (labeled D, TψC, and anti-codon in Fig. 1-1), a variable loop, and the amino acid acceptor stem ending with a single stranded cytidine-cytidine-adenosine (CCA) sequence (Holley *et al.*, 1965; Soll and RajBhandary, 1995). This specific secondary structure is defined by intramolecular hydrogen bonding between specific Watson-Crick base pairs (Soll and RajBhandary, 1995). The 'L-shaped' tertiary structure of a tRNA (Fig.1-2) is formed by the stacking of the acceptor stem onto

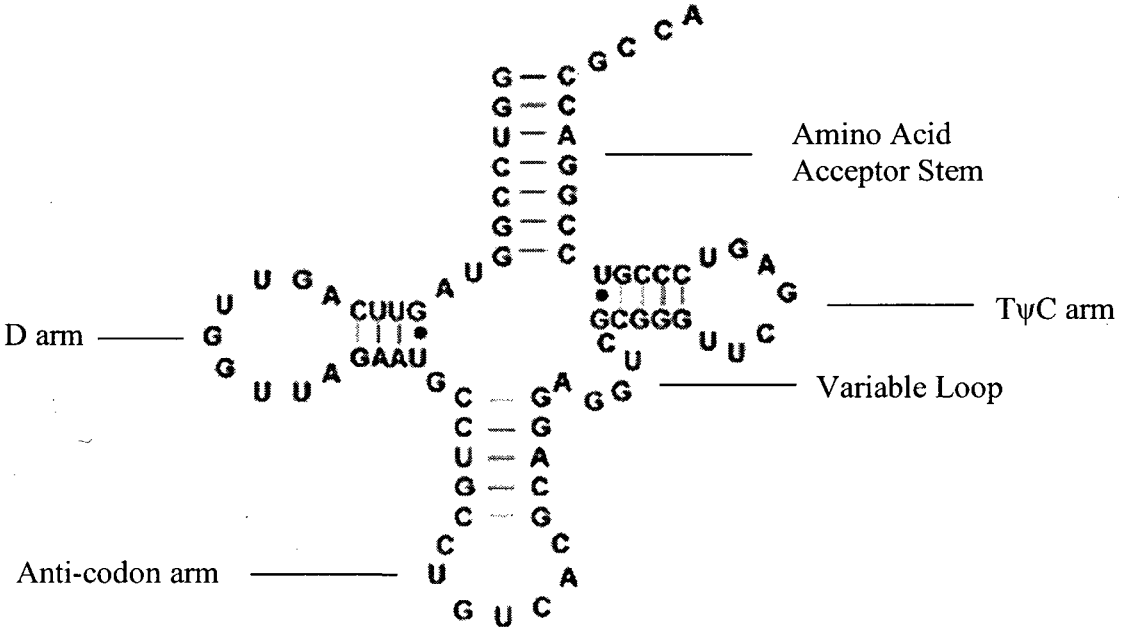

Fig. 1-1. The secondary structure of transfer RNA. This example is the $tRNA^{Asp}$ from *Bacillus subtilis*. Dashes (-) represent standard Watson-Crick base pairs, dots (●) indicate non-standard base pairs. (Modified from Sun *et al.*, 2006).

the TψC stem and the D stem onto the anti-codon stem through additional hydrogen bonding interactions (Soll and RajBhandary, 1995). In this native configuration the anti-codon loop is at one end of the molecule and the acceptor stem is at the other.

The process of making a functional tRNA begins with transcription of the gene coding for the tRNA, creating a primary transcript (King *et al.*, 1986; Deutscher, 1984; Burdon, 1971). Maturation of this primary transcript requires 'trimming' by 5' and 3' nucleases to remove excess nucleotides from the extremities, the removal of any introns (if present) by endonucleases, and modification of certain nucleotide residues (King *et al.*, 1986; Deutscher, 1984; Deutscher, 1990). The maturation process in some organisms

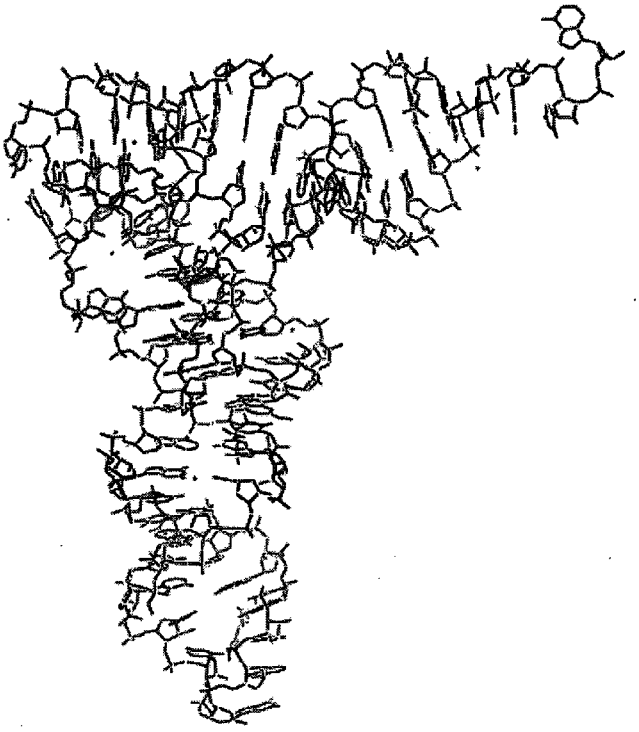

Fig. 1-2. The tertiary structure of transfer RNA (Jovine *et al.* 2000). Crystal structure of yeast phenylalanine transfer RNA at 2.0Å resolution. PDB file:1evv

also requires the addition of CMP, CMP, and AMP residues to positions 74, 75, and 76 at the 3'-terminus of the tRNA by ATP(CTP):tRNA nucleotidyltransferase (Deutscher, 1984; Sprinzl *et al.*, 1987; Deutscher, 1990). It is the activity of this enzyme that will be addressed here.

1.2 Transfer RNA nucleotidyltransferase

Transfer RNAs must contain an intact 3' terminal cytidine, cytidine, adenosine sequence for aminoacylation to occur at the final adenosine nucleotide (Hecht *et al.*, 1959). As mentioned previously, tRNA nucleotidyltransferase is responsible for the addition of this sequence to tRNAs that either are completely devoid of these residues (Deutscher, 1983), or require repair when the 3' ends are damaged (Deutscher, 1982; Deutscher, 1990). While all enzymes responsible for nucleotide addition are members of the nucleotidyltransferase superfamily, unlike enzymes such as poly(A) polymerase and terminal deoxynucleotidyltransferase (TdT) which are able to add strings of adenosine or deoxythymidines of various lengths without the use of a nucleic acid template (Yue *et al.*, 1996; Li *et al.*, 2002), tRNA nucleotidyltransferase adds a specific nucleotide sequence: cytidine, cytidine, adenosine (Yue *et al.*, 1996; Li *et al.*, 2002). This ability of tRNA nucleotidyltransferase to add a defined nucleotide sequence without the use of a nucleic acid template is what makes this enzyme particularly interesting to study.

1.3 Nucleotidyltransferase superfamily

The nucleotidyltransferase superfamily (previously referred to as the Polβ-nucleotidyltransferase superfamily) contains multiple families of nucleotidyltransferases with structural and functional similarity (Holm and Sander, 1995; Yue *et al.*, 1996;

Aravind and Koonin, 1999). This superfamily is characterized by the signature motif hG[G/S]$x_{9\text{-}13}$Dh[D/E]h in the active site sequence where uppercase letters are invariant, 'x' is any amino acid and 'h' is a hydrophobic amino acid (Holm and Sander, 1995; Martin and Keller, 1996; Avarind and Koonin, 1999). All enzymes in this superfamily use a nucleoside triphosphate (NTP) as substrate. The NTP undergoes a nucleophilic attack by a free hydroxyl group at the end of a polynucleotide which ultimately eliminates pyrophosphate from the original NTP substrate (Holm and Sander, 1995). While all nucleotidyltransferases have a conserved DxD motif (Holm and Sander, 1995) at the catalytic site required for binding the two essential divalent metal ions (Martin and Keller, 1996), the nucleotidyltransferase superfamily can be subdivided into two classes based on other specific sequence motifs in the catalytic domain (Yue *et al.*, 1996). Class I enzymes include archeal tRNA nucleotidyltransferases, DNA polymerase β, and eukaryotic poly(A) polymerases. Class II enzymes include eubacterial and eukaryotic tRNA nucleotidyltransferases and eubacterial poly(A) polymerases (Yue *et al.*, 1996). While the class II enzymes show sequence similarity in their amino terminal 25 kDa portions (Fig. 1-3), there is no evident conservation of sequence in the corresponding regions of the class I enzymes.

```
SC   ------------------MLRSTISLLMNSAAQKTMTNSNFVLNAPKIT----------   31
CG   ----------------------------------MFKAIRRVFTMIPRIQ----------   16
KL   ---------------------------------------MFKMVASKIQ----------   10
LA   ----------------MRLSFKTVTNVVVVLPRGRTRSIINFTLFPTITSNLVLHPLLRT   44
AT   ----------MRLSSLPINTLINLPKSLFLISPFRFRNLNRSLTVASRISSTLLRVSGVS   50
HS   ----------------MLRCLYHWHRPVLNRRWSRLCLLKQYLFT---------------   29
BS   ------------------------------------------------------------
AA   ------------------------------------------------------------
EC   ------------------------------------------------------------
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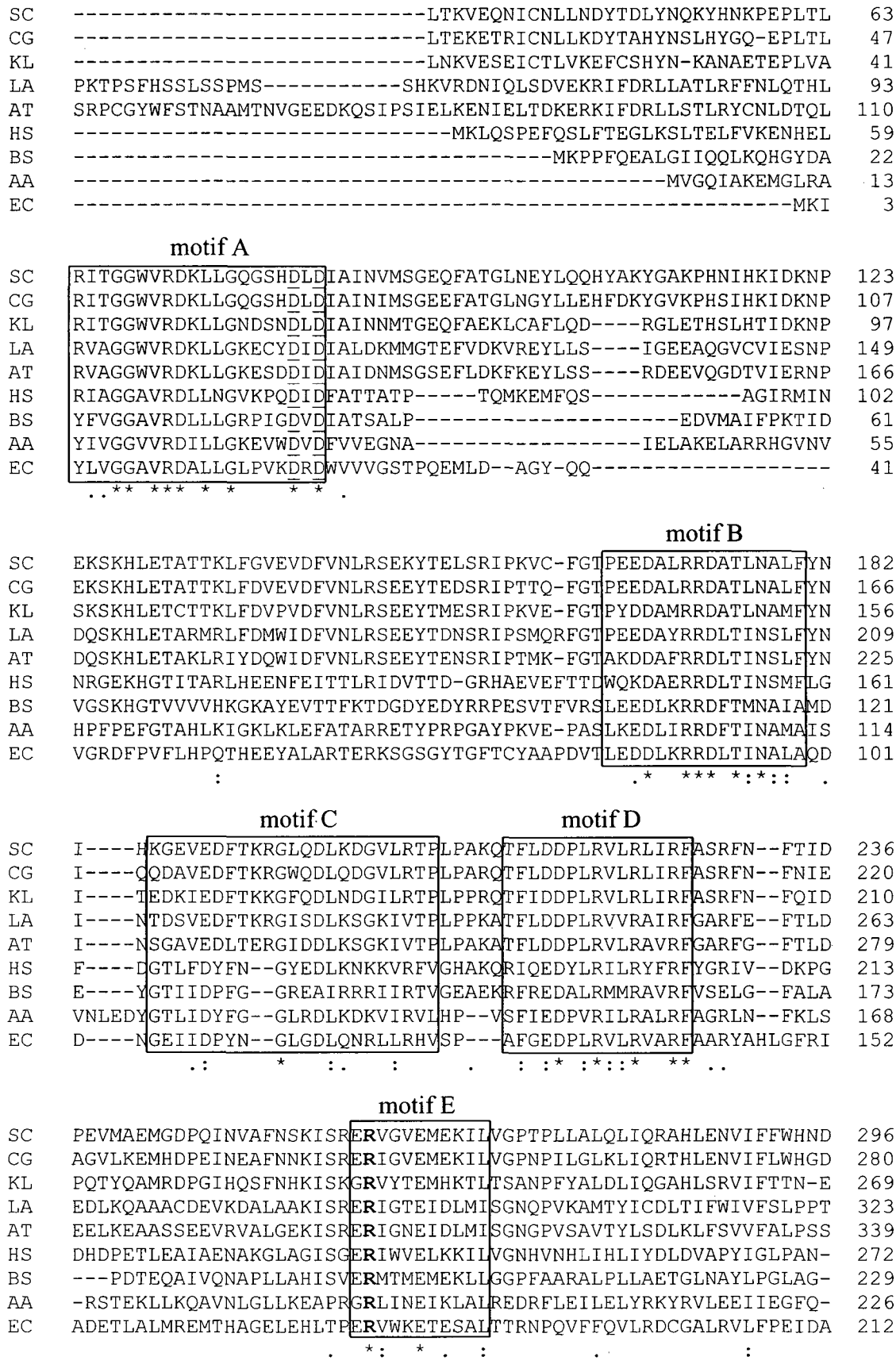

motif A
motif B
motif C
motif D
motif E

```
SC   SSVV------KFNEENCQDMDKINHVYNDNILNSHLKSFIELYPMFLEKLPILREKIGRS  350
CG   QSVI------EYNRKNWPQTKDVEDIYKKGIFNHHLKNFIHHYKDFLSRYLKLRQAIETK  334
KL   SS--------------PEIESIYEN--LDQ---HLKSVVETIPKLLKSHTTFASVFPGM  309
LA   FEPAISDGCERLCISQLDISWNLIHLLGKTTFTDEQRRLTLYAAMFLPLRNTIYREKKAK  383
AT   AEPSPPENCGSLSQSYLEAMWSLLKTPRPGKFSGEQRRLALYAAMFLPFRKTVYKDTKGK  399
HS   ----------------------------ASLEEFDKVSKNVDGFSPKPVTLLASLFKV  302
BS   ---------------------------------KEKQLRLAAAYRWPWLAAREERWAL  254
AA   ----------------------------------------WNEKVLQKLYALRKVVDWH  245
EC   LFG---------------------------VPAPAKWHPEIDTGIHTLMTLSMAAMLSP  244

SC   P-GFQQNFILSAILSPMANLQIIGNPKKKINNLVSVTESIVKEGLKLSKNDAAVIAKTVD  409
CG   DKSFQQNFLLASILIPMADLKIIALPKKKLNNTLPVSESIVREGLKFNKASSIVVARCVE  394
KL   QEPLILSLVLSGFKG----LKGPDPAKPK--NSIPLAGVITKEGLNFPNTQVDNVIACVE  363
LA   KVPVVNYIFRESLKRKAKDPETVLDLHRASNKFLSLIPCLVSN--EDVQIVGHDWMTELI  441
AT   SIPVVNHIFKFSMKRKTSDAETVMNIHQTTERFRSLIPSLEVK--KDVELDELTWAADIL  457
HS   QDDVTKLDLRLKIAKEEKNLGLFIVKNRKDLIKATDSSDPLKP-----------------  345
BS   LCHALGVQESRPFLRAWKLPNKVVDEAGAILTALADIP----------------------  292
AA   ALEFSEERIDYGWLYLLILISNLDYERGKHFLEEMSAPSWVRET----------------  289
EC   QVDVRFATLCHDLGKGLTPPELWPRHHGHGPAGVKLVEQLCQR-----------------  287

SC   SICSYEEILAKFADRSQLKKSEIGIFLRNFNGEWETAHFASLSDAFLKIPKLETKKI---  466
CG   NIAAYNSMVEKYLQSGDLKRSEVGTFLRELRGDWEIVHYVSLMDQYLKYISRKDNVV---  451
KL   SEDSYHNLVKNGKS---MKRSELGFALRKLGKNWQMVHFYNLCLDYLRHGDEP-------  413
LA   D-----------VPVSSRVRVLTGFLLRELRDFWRVALLISILLHPIDVNDTEDESS---  487
AT   EHWKSITLNDPVIPATSKIRVLTGFLLRDIKDFWRVSLLTSLLLSATVDGSNDHQDIGQL  517
HS   --------------------------YQDFIIDSREPDATTRVCELLK------------  367
BS   -------------------RPEAWTNEQLFSAGLERALSVETVRAAFTGAPPGP------  327
AA   -------------------YKFMKFKLGSLKEELKKAKENYEVYRLLKP-----------  319
EC   -------------------LRVPNEIRDLARLVAEFHDLIHTFPMLNPKTIVKLFDSID  327
                                 :

SC   ----ELLFQNYNEFYSYIFDNNLNNCHELKPIVDGKQMAKLLQMKPGP-WLGKINNEAIR  521
CG   ----NIIDK-YDRFWNYIQEQNLQDSDKMVPIIDGKRMVKILETKPGP-WLGKINDEVIL  505
KL   -------IPHYDEFYKHVHDCKLDDVYTLKHIINGKELAKLLDRKPGI-WMGETLDRILI  465
LA   --QLSKRRDLFNTVENSVIKLGLEKVWDVKQLINGKDVMSVLQLKGGP-MVKEWLDKAMA  544
AT   DFQLERMRETYLTVEATIHELGLDKIWDAKPLVNGREIMQIAELKGGSRLIREWQQKLLT  577
HS   -------------YQGEHCLLKEMQQWSIPPFPVSGHDIRKVGISSGKEIGALLQQLREQ  414
BS   -----------WHEKLRRRFASLPIKTKGELAVNGKDVIEWVGKPAGPWVKEALDAIWRA  376
AA   -------------LHTSVLLLLMLEEELKEKIKLYLEKLRKVKLPKEKIEELKKQGLKGK  366
EC   AWRKPQRVEQLALTSEADVRGRTGFESADYPQGRWLREAWEVAQSVPTKAVVEAGFKGVE  387

SC   WQFDNPTGTDQELITHLKAILPKYL---  546
CG   WQFDHPQGTEQELISFIKSILPNYLQ--  531
KL   WQLDNPDISKETFIENLNDIVHLP----  489
LA   CNLPIPQELQRNVLIG------------  560
AT   WQLAYPNGTAEECKEWMRDIKAKRQRIE  605
HS   WKKSGYQMEKDELLSYIKKT--------  434
BS   VVNGEVENEKERIYAWLMERNRTREKNC  404
AA   ELGERIEELKREIMNKIKLAAALE----  390
EC   IREELTRRRIAAVASWKEQRCPKPE---  412
```

Fig. 1-3. Multiple sequence alignment of selected class II tRNA nucleotidyltransferases. Sequence alignment generated by ClustalW (ClustalW server: Larkin *et al.*, 2007) of selected class II tRNA nucleotidyltransferases (accession numbers from Genbank in brackets). SC, *Saccharomyces cerevisiae* (AAFW02000048.1); CG, *Candida glabrata* (CAG62257); KL, *Kluyveromyces lactis* (AAG00316); LA, *Lupinus albus* (AAB03077); AT, *Arabidopsis thaliana* (NM_102113.3); HS, *Homo sapiens* (AB063105); BS, *Bacillus stearothermophilus* (Q7SIB1); AA, *Aquifex aeolicus* (1VFG_B); EC, *Escherichia coli* (AAA23541). Numbers indicate amino acid position within the protein sequence, (*) Indicates an amino acid conserved in all sequences, (:) indicates similar amino acids and (.) weakly similar amino acids. Boxed sequences represent conserved motifs A to E identified by Li *et al.* (2002). The arginine residue corresponding to *C. glabrata* Arg244 is in bold. Two conserved aspartic acid residues required for metal ion binding at the active site (Holm and Sander, 1995; Martin and Keller, 1996) are underlined.

1.4 Structural properties of tRNA nucleotidyltransferase

Since the first reported purification of tRNA nucleotidyltransferase from rat liver (Canellakis and Herbert, 1960), numerous tRNA nucleotidyltransferases from unicellular and multicellular organisms have been isolated and characterized (Carre *et al.*, 1970; Deutscher, 1972a; Dullin *et al.*, 1975; Poblete *et al.*, 1977, Schofield and Williams, 1977; Cudny *et al.*, 1978, Chen *et al.*, 1990, Shanmugam *et al.*, 1996). These enzymes range in size from 30 kDa to 70 kDa and all catalyze the same reaction (Deutscher, 1983).

Based on the available crystal structures, class II tRNA nucleotidyltransferases have been said to resemble a sea horse with a head, neck, body, and tail region (Fig. 1-4) (Li *et al.*, 2002). Studies have determined that tRNA nucleotidyltransferase contains a single active site in which CTP, ATP, and the 3' end of the tRNA are bound and processed (Yue *et al.*, 1998; Li *et al.*, 2002). The catalytic domain is contained within the head portion where the two divalent metal ions are bound by the conserved carboxylates (Li *et al.*, 2002). The neck domain confers the specificity with regard to nucleotide selection and addition (Li *et al.*, 2002). The binding of tRNA is believed to occur in the

body and tail regions of the enzyme based on alkylation studies with ethylnitrosourea and UV irradiation (Shi *et al.*, 1998). Furthermore, domain shuffling experiments between the C-terminal regions of *E. coli* tRNA nucleotidyltransferase and poly(A) polymerase have demonstrated that tRNA is anchored in the C-terminal region, restricting polymerization to three nucleotides (Betat *et al.*, 2004). This is also supported by cross-linking studies which have shown that tRNA does not translocate during CCA addition (Shi *et al.*, 1998), and has led to the current model requiring the refolding of the 3'-end of the tRNA after each nucleotide addition (Yue *et al.*, 1998).

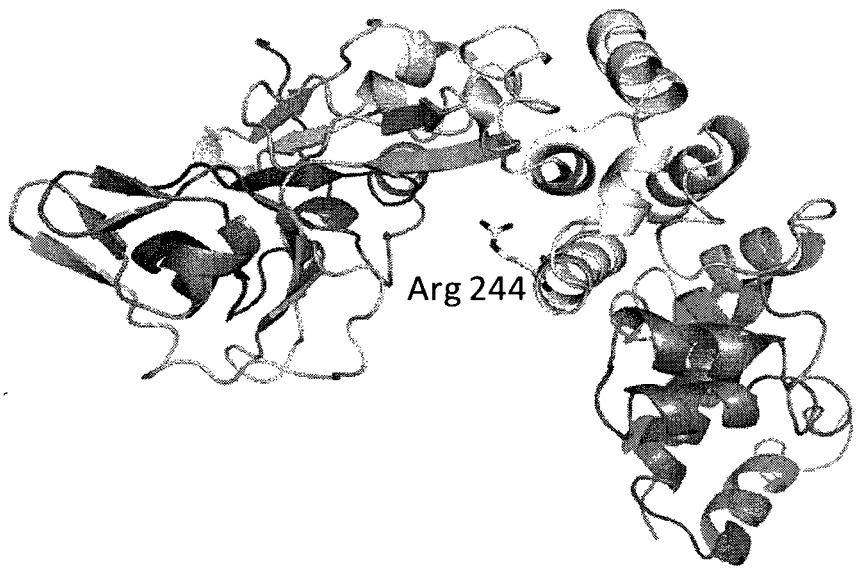


Fig 1-4. Model of *C. glabrata* tRNA nucleotidyltransferase The *C. glabrata* primary sequence was compared to the amino acids sequences contained in the Imperial College Protein Homology/analogY Recognition Engine (PHYRE) server (Bennett-Lovsey *et al.*, 2008). This program then created a model based on the determined crystal structure of the closest homologous sequence, *H. sapiens*. The model was then viewed with PyMol (DeLano, 2009). Arginine residue 244 is shown and labelled.

To date there are solved crystal structures for one class I (*Archaeglobus fulgidus* (Xiong *et al.*, 2003; Okabe *et al.*, 2003; Xiong and Steitz, 2004, Tomita *et al.*, 2006)) and three class II (*Aquifex aeolicus* (Tomita *et al.*, 2004), *Bacillus stearothermophilus* (Li *et al.*, 2002), and *Homo sapiens* (Augustin *et al.*, 2003)) tRNA nucleotidyltransferases. Of the three class II enzymes it is important to note that the *Aquifex aeolicus* structure is that of an adenosine-adding enzyme (Tomita *et al.*, 2004) as this organism requires two enzymes, one to add CC and another to add the terminal A, to complete the 3'-terminal sequence (Tomita *et al.*, 2001).

Although both class I and class II tRNA nucleotidyltransferases have evolved nucleotide selection through specific amino acid interactions, thereby eliminating the need for a nucleic acid template, they do this in different ways. Class I CCA-adding enzymes require a bound tRNA to specifically bind CTP and ATP in collaboration with a conserved arginine residue that bonds with the O2 and N3 positions of the CTP base and N1 of the ATP base (Toh *et al.*, 2008). In contrast, class II enzymes can bind both nucleotides specifically without the presence of tRNA through a dynamic protein template with interactions between the incoming bases and amino acid side chains mimicking Watson-Crick interactions (Li *et al.*, 2002). This requires that two of the three templating amino acids rotate after CTP addition to accommodate and add the final nucleotide, ATP (Li *et al.*, 2002). In both classes however, the protein helps refold the 3' terminus of the tRNA after each nucleotide addition to properly orient the terminal 3'OH for nucleophilic attack on the next incoming nucleotide. This allows for the same active site to be used for the incorporation of each nucleotide (Li *et al.*, 2002; Xiong and Steitz, 2004; Tomita *et al.*, 2006).

1.5 Substrate recognition by class II tRNA nucleotidyltransferases

Sequence alignments of class II enzymes in conjunction with analysis of solved crystal structures have revealed several highly conserved motifs (Fig. 1-3) (Li *et al.*, 2002). Motif A facilitates the binding of the nucleotide substrate by interacting with the triphosphate moiety through conserved glycine and arginine residues in a helix-turn structure and contains two acidic amino acids involved in catalysis (Li *et al.*, 2002). Motif B allows the enzyme to discriminate between deoxyribonucleotides and ribonucleotides through the base pairing of the second arginine group in the conserved signature sequence, RRD (Shanmugam *et al.*, 1996), with the 2' OH of the incoming ribonucleotide (Li *et al.*, 2002). The aspartate residue in this RRD motif is absolutely required for ATP addition but not CTP addition (Seth *et al.*, 2002). Motif C connects the head and neck regions and although conserved residues within this motif have yet to be assigned roles (Li *et al.*, 2002), the change of a conserved acidic residue within this motif rendered the *S. cerevisiae* enzyme temperature-sensitive without the loss of thermal stability at 37°C, suggesting a role in enzyme activity (Aebi *et al.*, 1990; Shan *et al.*, 2008).

The signature sequence (E/D)DxxR in motif D forms part of a dynamic protein template that allows the enzyme to recognize and bind the incoming nucleotides, while two additional conserved arginine residues serve to neutralize the phosphate moiety of the bound nucleotide (Fig. 1-5) (Li *et al.*, 2002). The aspartate and arginine residues in the (E/D)DxxR motif form Watson-Crick-like hydrogen bonding interactions with the incoming bases (Li *et al.*, 2002) while the conserved glutamate serves to stabilize the arginine residue through hydrogen bonding. Specifically, the carboxylate of the aspartate

forms hydrogen bonds with the amino group at position four of CTP or at position six of ATP while the guanidinium group of the arginine residue hydrogen bonds with the nitrogen and oxygen atoms at positions three and two respectively of an incoming CTP. A crucial feature in the mechanism of this reaction is the rotation of both the arginine and glutamate residues after the first two CTP molecules are added to the tRNA template

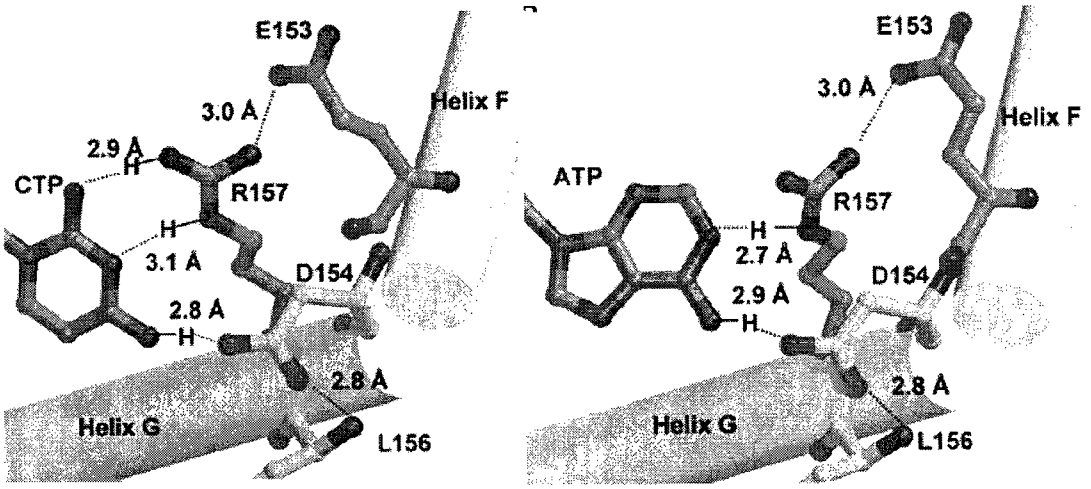


Fig. 1-5. A model, based on the *B. stearothermophilus* enzyme, of the Watson-Crick-like base pairing between the EDxxR motif and the nucleotide substrates (Modified from Li *et al.*, 2002). Left: Hydrogen bond donating interactions provided by the guanidinium group of the arginine as well as a hydrogen bond donating interaction provided by the aspartate residue allows the enzyme to bind CTP. Right: The same residues provide the same donor-acceptor interactions to bind ATP, however, the positions of the arginine and glutamate residues have changed to provide one hydrogen bond from the guanidinium group instead of two.

such that the arginine can hydrogen bond with the nitrogen at position one of ATP (Fig. 1-5). This allows the template to form three and two bonds with the incoming CTP and ATP molecules, respectively. Due to the specific donor-acceptor pattern within the Watson-Crick pairing (Fig. 1-6), UTP and GTP are excluded.

Motif E has two conserved residues in class II enzymes: an arginine (represented by Arg244 in *C. glabrata*) and a glutamate. These residues are situated in helix J (Li *et*

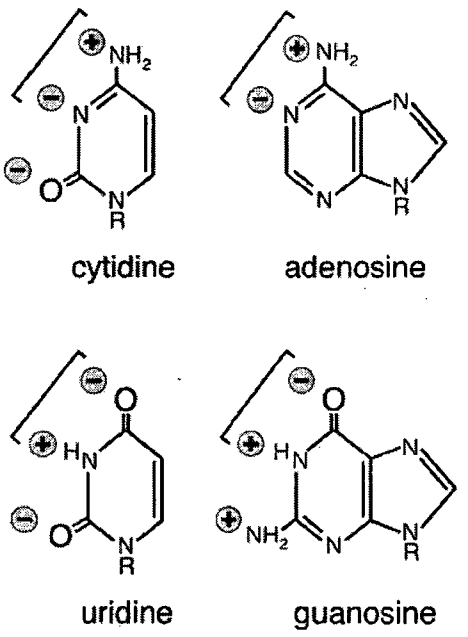


Fig 1-6. Hydrogen bond donor-acceptor patterns of the four bases (Cho *et al.* 2003). The tRNA nucleotidyltransferase recognizes CTP and ATP and discriminates against UTP and GTP through the use of hydrogen bonding interactions between the base and the amino acid template EDxxR. 'Base pairing' occurs at the 3 and 4 positions of pyrimidines and the equivalent 1 and 6 positions of purines. Hydrogen bond donors are indicated by (+) and hydrogen bond acceptors are indicated by (-).

al., 2002) and are believed to interact and stabilize the residues in domain D (helix G) which contain the dynamic protein template (Li *et al.*, 2002). Furthermore, it has been suggested that the arginine residue in motif E may interact with the growing 3' end of the tRNA (Cho *et al.*, 2007). Both the arginine and glutamate residues in motif E have been shown to be involved in limiting the extension of the 3' terminus to three nucleotides as well as conferring nucleotide specificity in the *B. stearothermophilus* enzyme (Cho *et al.*, 2007).

1.6 Rationale

In the current study, the role of arginine 244 in the *Candida glabrata* tRNA nucleotidyltransferase will be explored. As described above, this arginine residue which lies within motif E and is conserved in all class II tRNA nucleotidyltransferases (Fig. 1-3) is believed to be important to the functioning of the enzyme. Models based on known class II tRNA nucleotidyltransferase crystal structures (Cho *et al.*, 2007) suggest a role for arginine 244 in stabilizing, through hydrogen bonding, the position of the second acidic residue in the (E/D)DxxR protein templating sequence in domain D (Fig. 1-7). Another potential role for this residue may be to stabilize or coordinate the growing 3' end of the tRNA substrate in the active site, possibly forming interactions through hydrogen bonding with the phosphate group of the first added cytidine base (Fig. 1-7). Finally, this arginine residue situated in helix J, may act through a network of hydrogen bonds to assist both in nucleotide binding and nucleotide addition to the 3' end of tRNA, acting as a keystone in substrate binding and enzyme specificity.

To determine if the arginine residue at position 244 was indeed of interest, site-directed mutagenesis was performed in the *Candida glabrata CCA1* gene coding for tRNA nucleotidyltransferase. Substitution of arginine at this position by alanine, methionine, or lysine resulted in cell death (Fig. 1-8) in *in vivo* assays in yeast cells, indicating a vital role for this arginine residue. In this study, the role of arginine 244 in *Candida glabrata* tRNA nucleotidyltransferase structure and function will be explored by a combination of biophysical and biochemical approaches. Potential alterations in secondary and tertiary structure will be determined by circular dichroism and

fluorescence spectroscopy and effects on enzyme activity will be assessed using *in vitro* enzyme assays.

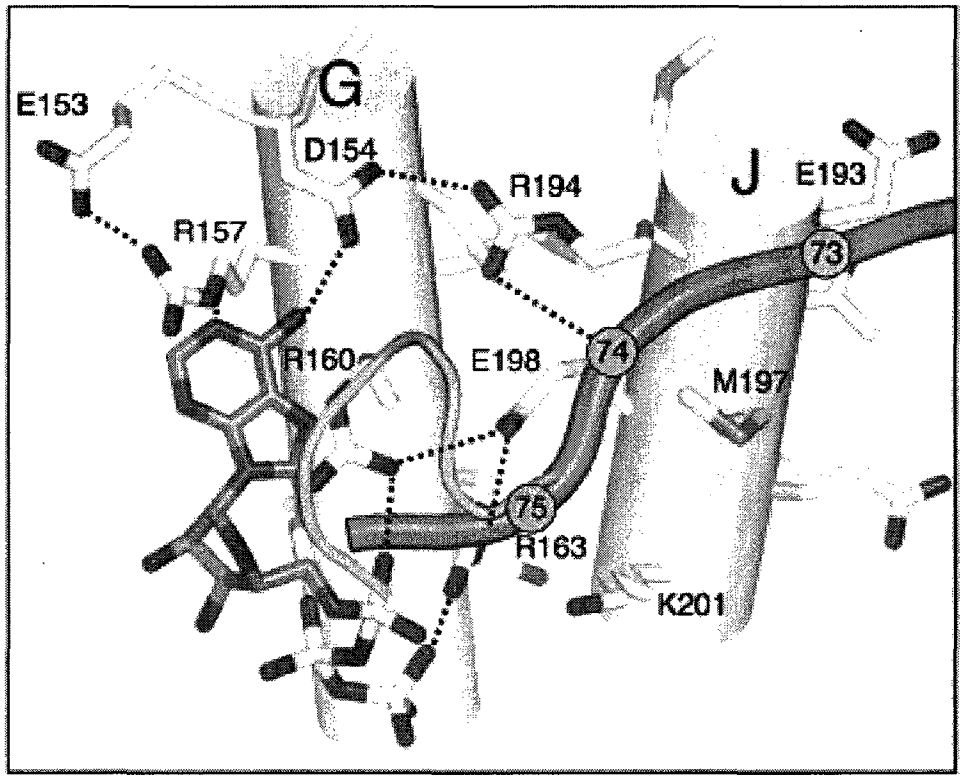


Fig. 1-7. Model of helix G and helix J with incoming ATP and the growing 3' end of a tRNA (Cho *et al.* 2007). The model is based on the crystal structure of *A. aeolicus* A-adding enzyme, however the numbering of the amino acids corresponds to the homologous residues from the *B. stearothermophilus* enzyme. The 3' end of the tRNA with positions 73-75 are highlighted corresponding to the discriminator base N73, and position one and two of the C74C75A76 sequence. A β-turn is also shown in the model and may interact with the growing 3' end of tRNA-NCC. R194 corresponds to Arg244 in the *C. glabrata* enzyme.

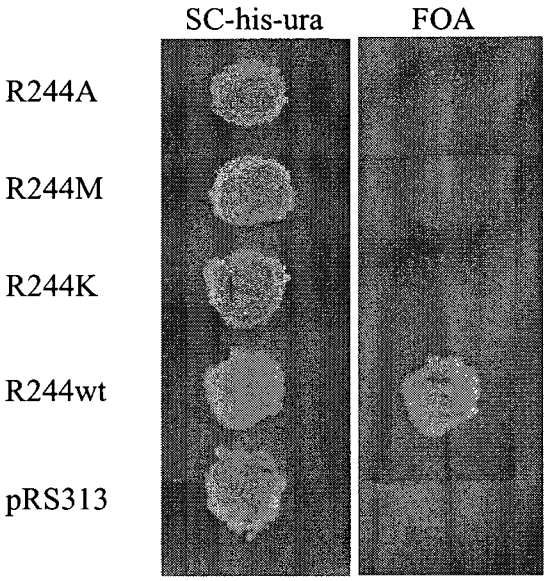


Fig. 1-8. Effect on viability of substitutions at position 244 of *C. glabrata* tRNA nucleotidyltransferase (Dr. Pamela J. Hanic-Joyce, personal communication). *Candida glabrata* strain GCP1-2 lacking its own *CCA1* gene and carrying the *Saccharomyces cerevisiae CCA1* gene on plasmid pRS316 was transformed with pRS313 derivatives bearing *Candida glabrata CCA1* genes coding for native (wt) or R244 variant (R244A, R244M, and R244K) tRNA nucleotidyltransferases. Transformants were grown on synthetic complete medium lacking histidine and uracil (SC-his-ura) and were replica-plated to medium containing 5-fluoro-orotic acid (FOA). Plates were incubated for 3 days at 30°C.

2. Materials and Methods

2.1 Strains, Buffers, Growth Media, and Solutions

The *E. coli* strains (XL2-Blue and BL21(DE3)) were purchased from Stratagene. Growth media and buffers used in this study are listed in Table 2.1. Chemicals and their grade of purity can be found in Table 2.2

Table 2-1 Recipes for buffers, growth media, and solutions

Buffers, Growth Media, and Solutions	Components
YT (Sambrook *et al.*, 1989)	0.5% Yeast Extract, 0.8% Tryptone, 0.5% NaCl
Easy Prep. Buffer (Berghammer and Auer, 1993)	10 mM Tris-HCl pH 8, 1 mM EDTA, 15% sucrose, 2 mg/mL lysozyme (Sigma-Aldrich), 0.1 mg/mL BSA acetylated (Fermentas), 0.1 mg/mL RNaseA (Boiled)
1X Phosphate-buffered saline pH7.4 (PBS) (Sambrook *et al.*, 1989)	8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4
5X TBE (4L) (Modified from Sambrook *et al.*, 1989)	120 g Tris, 62 g boric acid, 40 mL 0.5 M EDTA (pH8)
TEG solution (Promega, 1991)	25 mM Tris-HCl (pH 8.0), 10mM EDTA, 50mM glucose
Lysis buffer (for protein purification) (Modified from Shan, 2005)	1X PBS pH7.4, 1mM EDTA, 0.01% Lysozyme (Sigma-Aldrich), 1 tablet « Complete Mini » Protease Inhibitor Cocktail (Roche) (per 50 mL)
30% acrylamide solution (29:1) (Sambrook *et al.*, 1989)	29.2 g acrylamide, 0.8 g bis-acrylamide, to 100 mL in dH_2O
5X SDS Running Buffer (Modified from Sambrook *et al.*, 1989)	72 g/L glycine, 15 g/L Tris-HCl, 5 g/L sodium dodecyl sulfate (SDS)
SDS-Polyacrylamide stacking gel (Sambrook *et al.*, 1989)	1.3 mL 30% acrylamide solution, 6.1 mL dH_2O, 2.5 mL 0.5 M Tris-HCl pH 6.8, 100 µL 10% sodium dodecyl sulfate, 100 µL 10% ammonium persulfate (APS), 10 µL TEMED
13% SDS-Polyacrylamide resolving gel (Sambrook *et al.*, 1989)	4.3 mL 30% acrylamide solution, 3 mL dH_2O, 2.5 mL 1.5 M Tris-HCl pH 8.8, 100 µL 10% sodium dodecyl sulfate, 100 µL 10% ammonium persulfate, 10 µL TEMED
SDS-PAGE staining and destaining solutions: (Wong *et al.*, 2000)	
Staining solution A,	25% isopropanol, 10% acetic acid, 0.05% Coomassie brilliant blue
Staining solution B,	10% isopropanol, 10% acetic acid, 0.005% Coomassie brilliant blue
Destaining solution	10% isopropanol, 10% acetic acid

Peatties loading buffer (Peattie, 1979)	10 M urea, 5 mM Tris-borate pH8.3, 0.1 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue
12% (20%) Polyacrylamide 7 M Urea denaturing gel	11.4 g (19 g) acrylamide, 0.6 g (1 g) bis-acrylamide, 42 g urea, 650 µL 10% ammonium persulfate, 20 µL TEMED. Bring to 100 mL with dH_2O

Table 2-2. List of chemicals

Chemical	Grade	Manufacturer
Potassium phosphate monobasic		Anachemia
Calcium chloride ($CaCl_2$)		
Bio-tryptone	Bacteriological	Bioshop
Yeast Extract (pH 7.0 ± 0.5)		
Sucrose	Ultra pure	
ethylenediaminetetraacetic acid (EDTA)	Biotechnology	
Acrylamide		
Bis-Acrylamide		
Tris	Bio ultra pure	
Glycine	Biotechnology	
Ammonium Persulfate (APS)		
Agarose	Biotechnology	
Tetramethylethylenediamine (TEMED)		
Coomassie brilliant blue R-250		
Urea	Bio ultra pure	
Sodium phosphate dibasic, heptahydrate	ACS	
D-glucose	Reagent grade	
Boric acid	Biotechnology	
Potassium chloride (KCl)		
Ampicillin (Amp)	Biotechnology	
Isopropyl-β-D-thiogalactopyranoside (IPTG)		
Ethanol (EtOH)	99%	Commercial Alcohols Inc.
Magnesium Chloride Hexahydrate, ($MgCl_2 - 6dH_2O$)		EM Science
Isopropanol		Fisher Scientific
Acetic acid		
Glycerol	Enzyme	
Sodium Chloride (NaCl)	Biological	

Bovine serum albumin acetylated		Fermentas
Nuclease-free water		Integrated DNA Technologies
Sodium dodecyl sulphate (SDS)	Ultra pure	MP Biomedicals
Polyethylene glycol (PEG)	Biotechnology	
Ethidium bromide (EtBr)	98%	
EcoLite scintillation fluid		
[α-^{32}P] adenosine triphosphate (ATP) (10 μCi/μL, 3000 Ci/mmol)		Perkin Elmer
[α-^{32}P] cytidine triphosphate (CTP) (10 μCi/μL, 3000 Ci/mmol)		
[α-^{32}P] guanosine triphosphate (GTP) (10 μCi/μL, 3000 Ci/mmol)		
Baker's yeast tRNA		Roche
<<Complete mini>> cocktail inhibitor		
Agar		Sigma-Aldrich

2.2 Generating the lysine tRNA nucleotidyltransferase variant in pGEX-2T

2.2.1 Site-specific mutagenesis

The *Candida glabrata CCA1* gene from strain CBS138 had been cloned and sequenced previously in this lab (Hanic-Joyce and Joyce, 2002). This gene was inserted into the expression plasmid pGEX-2T (GE Healthcare) by Paul B. M. Joyce and site-directed mutagenesis by QuikChange™ mutagenesis (Stratagene) was performed to generate mutants coding for alanine or methionine in place of arginine 244. In the present study the same technique was used to change that arginine residue to lysine. The following oligonucleotides were used in mutagenesis:

fR244KCfr9I
5'-ATAAGATATCCCG**G**GAGA**A**AATTGGTGTGGAGATGG-3'

rR244KCfr9I
5'-CCATCTCCACACCAATT**T**TCTC**C**CGGGATATCTTAT-3'

Point mutations are in bold and a newly created *Cfr*9I restriction site is underlined. To generate the mutant genes, an initial cycle of 96°C for 2 minutes (to denature the double stranded DNA), 45°C for 1 minute (to allow the oligonucleotides to anneal), and 68°C for 16 minutes (to extend the new DNA strands) was followed by 18 cycles with the same parameters except that the melting step (96°C) was reduced from 2 minutes to 30 seconds. Each reaction contained 100 ng of template, 10 pmol of each primer, 0.2 mM of each deoxynucleotide, 5 µL of 10X Pfu buffer, and 1 µL (10 units) Pfu DNA polymerase (Fermentas). At the completion of the reaction 1 µL (10 units) *Dpn*I (Fermentas) was added to the reaction tube with incubation in a 37°C water bath for two hours to digest the original template DNA. After restriction digestion, 6 µL of 3 M NaOAc (pH 4.6) and 120 µL of 99% EtOH, were added with subsequent vortexing and incubation at -70°C for an hour to precipitate the DNA. After incubation the samples were centrifuged at 16 000 xg at 4°C for 30 minutes, the supernatant removed, the pellet was washed with 80% EtOH, and centrifuged again for 5 minutes. The pellet was then dried and resuspended in 15 µL of sterile dH_2O. Typically, 7.5 µL of the resuspended DNA was used for transformation (see section 2.2.3).

2.2.2 Preparation of competent *E. coli* cells (Capage and Hill, 1979; Dagert and Erhlich, 1979; Lederburg and Cohen, 1974).

E. coli was streaked for single colonies on a Petri plate containing YT medium and the plates were incubated at 37°C overnight. A single colony from the overnight plate was used to inoculate 5 mL of YT medium and this was incubated overnight on a Lab-Line Orbital Shaker at 37°C and 225 rpm. The next day these 5 mL were added to 400 mL of YT medium in a 1 L Erlenmeyer flask. The flask was shaken at 225 rpm and

incubated at 37°C in a New Brunswick Scientific Innova 4330 Refrigerated Incubator Shaker until the optical density, read at wavelength 600 nm (OD_{600}), reached between 0.6 and 0.7 absorbance units. The culture was chilled on ice for 10 minutes and centrifuged at 4400 xg at 4°C for 15 minutes. The supernatant was decanted and the pellet was resuspended in 200 mL of 0.1 M $MgCl_2$. Cells were centrifuged again as previously, but for 25 minutes. The supernatant was decanted and the cell pellet resuspended in 200 mL of 0.1 M $MgCl_2$ by gentle mixing. The flask was incubated on slushy ice for 25 minutes and the cells were centrifuged again, as above, for 25 minutes. The supernatant was decanted and the cell pellet resuspended to a final volume of 20 mL 0.1 M $CaCl_2$ and 14% glycerol. Aliquots (1 mL) of cells were distributed to twenty 1.5 mL microfuge tubes, incubated at 4°C overnight and then stored at -70°C until used.

2.2.3 *E. coli* transformation

Competent cells (100 µL) were incubated in a microfuge tube on ice with an appropriate amount of DNA for 10 minutes. The cells were heat-shocked for 45 seconds at 42°C and subsequently cooled on ice. Then, 100 µL of YT medium was added to the tubes with gentle mixing and incubation in a 37°C water bath for 2 hours. The cells were then plated on Petri plates containing YT medium plus 50 µg/mL ampicillin.

2.2.4 'Easyprep' plasmid isolation (Berghammer and Auer, 1993)

A single colony was selected from a YT+Amp plate and inoculated into 1.5 mL of YT + Amp liquid medium, grown overnight at 37°C, and the cells pelleted by centrifugation at 16 000 xg, at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended with 50 µL Easy Prep Buffer (Table 2-1)(Berhammer and Auer,

1993). Samples were shaken at room temperature for 10 minutes, boiled for 60 seconds and subsequently incubated on ice for 60 seconds. After centrifugation at 16 000 xg at room temperature for 15 minutes, the supernatant was transferred to a new microfuge tube and stored at -20°C.

2.2.5 Restriction analysis

Typically, DNA (5 µL aliquot from Easy Prep) was digested with 1 µL (10 units) of restriction enzyme for two hours at 37°C in the appropriate buffer (Fermentas) in a 20 µL reaction volume. For double digests the DNA was ethanol precipitated (as described above) after digestion with the first enzyme, and resuspended in 17 µL of distilled water prior to digestion with 1 µL (10 units) of the second enzyme and 2 µL of the appropriate 10X buffer as recommended by the supplier (Fermentas). For *SalI*/*Cfr*9I double digests the samples were digested first with *Sal*I and then with *Cfr*9I.

2.2.6 Agarose gel electrophoresis

Agarose was dissolved in 50 mL 1X TBE to the appropriate concentration and 4 µL of 10 mg/mL of ethidium bromide (EtBr) was added. Loading dye (Fermentas) was added to each sample and electrophoresis was carried out in 1XTBE buffer at 80V for one hour. The resulting bands were viewed on a FluorChem®FC2 ultraviolet light emitting box from Alpha Innotech.

2.3 Expression and purification of tRNA nucleotidyltransferase by GST-affinity chromatography

2.3.1 Protein expression

Eight flasks containing 1 L of YT + 50 μg/mL ampicillin medium were inoculated with 5 mL of an overnight culture containing *E. coli* BL21(DE3) carrying the plasmids of interest. The cultures were incubated in a New Brunswick Scientific Innova 4330 Refrigerated Incubator Shaker at 37°C and 225 rpm. *E. coli* cells were grown to an OD_{600} of 0.6-0.7 and induced with a final concentration of 1 mM IPTG. Induction was carried out at 18°C for 12-16 hours.

2.3.2 Cell lysis

Cells were centrifuged for 15 minutes, at 4°C and 4400 xg and the resulting pellets were frozen at -70°C for at least an hour. Pellets were thawed on ice and resuspended in cold lysis buffer (See Table 2-1). Lysis of cells was accomplished by sonication with a Branson Sonifier 250 from VWR Scientific or by a French®Pressure cell press from ThermoSpectonic. Sonication was carried out with the large tip at an output of 50 with 20 cycles of 10 seconds sonication plus 20 seconds cooling on ice. Cells which are lysed by French®Pressure cell were passaged at 1000 psi dropwise and the collected lysate passaged a second time using the same parameters.

2.3.3 Glutathione Sepharose Fast Flow 4B chromatography

Purification of GST-fusion proteins was done using Glutathione Sepharose Fast Flow 4B from GE Healthcare. Fresh resin (3 mL) was packed by gravity in a 1.5 cm x 10 cm Bio-Rad column. The column was rinsed with 10 bed volumes of distilled water and equilibrated with 10 bed volumes of 1X PBS (pH 7.4). After use, regeneration of the

resin involved washing the column with two bed volumes of 6 M guanidine-HCl, two bed volumes of 10 mM reduced glutathione in 1X PBS (pH 7.4), two bed volumes of 1% Triton X-100 and two bed volumes of 70% ethanol. Between washes the resin was rinsed with five bed volumes of 1X PBS (pH 7.4). When not in use the resin was stored in 20% ethanol.

2.3.4 Purification of GST-tagged tRNA nucleotidyltransferase and thrombin cleavage

After clearing the cell lysate by centrifugation at 39 000 xg at 4°C for 40 minutes it was loaded on the column by cycling overnight at 4°C at a flow rate of 1 mL/min using a Pharmacia LKB Pump P-1 peristalic pump. Protein that was not bound to the column was eluted with a minimum volume of 500 mL of 2X PBS (pH7.4), the spout of the column was plugged and 10 mL of 1X PBS (pH7.4) with 2.5 mM $CaCl_2$ was added to the column with gentle mixing. Subsequently, five units of thrombin (Amersham Biosciences) were added directly to the resuspended resin with incubation at 4°C overnight on a Barnstead rotisserie. The column was returned to the upright position and 1 mL fractions were collected and analyzed by SDS-PAGE (see section 2.3.5). Fractions containing tRNA nucleotidyltransferase were pooled, transferred to a 50 kDa Spectra/Por6 dialysis bag and the thrombin removed by dialysis in 4 L of 1X PBS (pH7.4) with two buffer changes, one every four hours. The volume was reduced to 1-4 mL utilizing an Amicon filtration unit under nitrogen gas with a 30 kDa Millipore ultrafiltration membrane. Finally, glycerol was added to a final concentration of 10% and the protein was divided into 20 µL fractions and stored at -70°C.

2.3.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970)

The resolving gel was prepared by mixing 4.3 mL of 30% (29:1 acrylamide/bisacrylamide) polyacrylamide solution, 3 mL dH_2O, 2.5 mL Tris-HCl (pH 8.8), 100 µL 10% sodium dodecyl sulfate, 100 µL 10% ammonium persulfate, and 10 µL TEMED. The mix was pipetted between two 4 cm by 10 cm glass plates separated by 4 mm spacers according to the instructions accompanying the Bio-Rad apparatus. Isopropanol (60%) was layered on top of the resolving gel to level the surface. Once the gel had polymerized, the isopropanol was removed and the stacking gel was added. The stacking gel was prepared by mixing 1.3 mL of 30% (29:1 acrylamide/ bisacrylamide) polyacrylamide solution, 6 mL dH_2O, 2.5 mL Tris-HCl (pH 6.8), 100 µL 10% sodium dodecyl sulfate, 100 µL 10% ammonium persulfate, and 10 µL TEMED and added to the apparatus. The appropriate comb was inserted and the gel allowed to polymerize. After adding loading buffer (Bio-Rad) the samples were boiled for 5 minutes, cooled on ice, and loaded onto the gel which was submerged in running buffer. Samples were allowed to separate at 80 volts for 15 minutes and then at 200 volts for ~1 hour. The gels were stained by a modification of the procedure of Wong *et al.* (2000). The gel was transferred to a vessel containing 50 mL of staining solution A and microwaved for 1 minute and 20 secs. The vessel containing the gel was shaken gently on an orbital shaker at room temperature for ≥5 minutes. The gel was rinsed with dH_2O and then transferred to a vessel containing 50 mL of staining solution B and the microwave/shaking procedure was repeated. The gel was rinsed again and transferred to another vessel containing 50 mL of destaining solution and a folded Kimwipe. The microwave/shaking procedure was repeated again. The gel was rinsed one last time, framed with cellophane, and left to dry.

2.3.6 Determination of protein concentration

Protein concentration was determined by the procedure of Smith *et al.* (1985) using the Pierce BCA™ Protein Assay Kit with bovine serum albumin (BSA) as the protein standard for this assay. A 200 µL sample was added to 1 mL of reagent A which was subsequently vortexed and incubated for 30 minutes at 60°C. Reactions were cooled to room temperature and the optical density was measured at 562 nm. The protein concentration of each sample was determined by plotting its absorbance value against the BSA standard curve.

2.4 Biophysical characterization of tRNA nucleotidyltransferase

2.4.1 Secondary structure determination by circular dichroism

The instrument used was a Jasco 815 circular dichroism spectrophotometer. The program used was 'Spectrum Measurement'. The spectrum settings on the spectrophotometer were set to scan over the region 260 nm to 200 nm with 5 accumulations using a bandwidth of 1 nm, a response of 0.25 seconds, sensitivity set to standard, data pitch at 0.2 nm, and a scanning speed of 20 nm/min. The cell length used was 0.1 cm in combination with a 5 mm spacer. The temperature was kept at 20°C with the use of a Pelletier water bath accessory. Flow rate was kept at 3 L/min of nitrogen. Aliquots of 200 µL of 0.262 mg/mL of protein (wild-type or variant) in 1X PBS (pH 7.4) were used for the spectrum measurements.

2.4.2 Temperature denaturation monitored by circular dichroism

The same instrument and flow rate were used as in section 2.4.1 and the program used was 'Variable Temperature'. The temperature denaturation scans were observed at

the monitor wavelength of 208 nm. The bandwidth used was 1 nm, response 0.25 seconds, sensitivity set to standard, data pitch at 0.2°C, and the temperature slope set to 15°C/hour. The start and end temperatures were 20°C and 95°C, respectively. The cell length used was 0.1 cm in combination with a 5 mm spacer. A 200 µL aliquot of protein at a concentration of 0.262 mg/mL in 1X PBS (pH 7.4) was used and the opening of the cuvette was sealed with parafilm to prevent volume loss due to evaporation.

2.4.3 Tertiary structure determination by fluorescence spectroscopy

The instrument used was a Shimadzu RF-5301PC spectrofluorophotometer. The parameters of the instrument were the following: the spectrum type: emission, the excitation wavelength: 280 nm, emission wavelength range: 295 nm to 400 nm, recording range: 0 to 600, scanning speed: fast, sampling interval: 1.0 nm, slit widths for excitation and emission: 5 nm, sensitivity: high and response time: auto. A 10 mM quartz cell was used with 500 µL of 0.02 mg/mL of sample in 1X PBS (pH 7.4).

2.5 Enzyme Activity Assays

2.5.1 Activity assays with Baker's Yeast tRNA (Modified from Cudny *et al.*, 1978)

Each standard assay was carried out in a final volume of 100 µL of a reaction mix containing 100 mM glycine buffer (pH9), 10 mM $MgCl_2$, 20 µM Baker's Yeast tRNA, 1 mM ATP, and 0.4 mM CTP, 0.2 µL [α^{32}P] ATP (10 µCi/µL, 3000 Ci/mmol) and 100 ng of protein for 2 minutes at room temperature. Each reaction was terminated by adding 100 µL of 2 M HCl and incubating on ice for 20 minutes. Precipitable counts were collected on prewetted (1 M HCl) GF/C filters on a Millipore sampling manifold under vacuum suction. Free [α^{32}P] ATP was rinsed off the filters with 20X 10 mL of 1 M HCl.

The filters were washed with 3X 10 mL of 99% EtOH and allowed to dry prior to transfer to scintillation vials containing 5 mL of EcoLite scintillation fluid. Samples were counted on a LKB WALLAC-1217 RackBeta liquid scintillation counter. In some cases the relative amounts of labelled, or unlabelled ATP or CTP was altered.

2.5.2 Large scale isolation of G73 and pmBSDCCA plasmid DNA from *E. coli* (Modified from Promega, 1991)

Plasmids G73 and pmBsDCCA were a generous gift from Alan Weiner (University of Washington) and originally constructed by Cho *et al.* (2002) and Oh and Pace (1994), respectively. Each plasmid contained a *B. subtilis* $tRNA^{Asp}$ gene modified to contain different restriction sites to produce specific 3'-ends after restriction digestion and *in vitro* transcription. YT medium (100 mL) containing 50 μg/mL ampicillin was inoculated with a single *E. coli* colony transformed with the desired plasmid. The culture was grown overnight in a New Brunswick Scientific Innova 4330 Refrigerated Incubator Shaker at 37°C at 225 rpm. Cells were pelleted by centrifugation at 5000 xg at 4°C for 15 minutes, the supernatant removed, and the cell pellet resuspended in 3 mL of TEG solution. The cells were incubated on ice for 10 minutes and the contents transferred to Oak Ridge tubes. Then, 6 mL of 0.2 N NaOH, 1% sodium dodecyl sulfate was added, and the contents mixed by inversion. Subsequently, 3.75 mL of 3 M sodium acetate (pH5.2) was added and mixed by inversion. The tube was incubated on ice for 20 minutes. The cells were pelleted by centrifugation at 12 000 xg at 4°C for 15 minutes, the supernatant was transferred to a new Oak Ridge tube and 5 μL of 10 mg/mL of DNase-Free RNase A was added. The tubes were incubated for 30 minutes in a 50°C water bath and the supernatant was extracted by adding an equal volume of phenol, vortexing, and

centrifuging at 12 000 xg at 4°C for 5 minutes. The supernatant was transferred to a new Oak Ridge tube and the phenol extraction was repeated. An equal volume of ether was added, the tube vortexed, and centrifuged as done previously for the phenol extraction. An equal volume of isopropanol was added, the tubes were vortexed, and incubated at -70°C for an hour. Plasmid DNA was pelleted by centrifugation at 12 000 xg at 4°C for 15 minutes, the supernatant was discarded, and the pellet was air-dried. The pellet was resuspended in 350 µL of distilled water, transferred to a 1.5 mL microfuge tube and 50 µL of 4 M NaCl and 110 µL of 30% PEG-8000 were added. The tubes were vortexed and allowed to stand on ice for 1 hour. Plasmid DNA was pelleted by centrifugation at 16 000 xg at 4°C for 20 minutes, the supernatant was discarded, 500 µL of 80% EtOH was added, and the tubes were centrifuged again at 16 000 xg at 4°C for 5 minutes. The supernatant was discarded and the pellet was air-dried. The plasmid DNA was resuspended with 450 µL dH_2O and 50µL 3 M NaOAc (pH5.2), followed by two extractions with 500 µL phenol and two with 500 µL ether as described previously. The aqueous phase was collected and the DNA precipitated with two volumes of 99% EtOH. Tubes were vortexed, incubated for 1 hour at -70°C, and pelleted by centrifugation for 30 minutes at 4°C and 16 000 xg. The pellet was washed with one volume of 80% EtOH and the tubes were centrifuged for 5 minutes, 16 000 xg at 4°C. Plasmid DNA was air-dried and resuspended in a desired volume of dH_2O. DNA was used for agarose gel electrophoresis (see 2.2.6) to confirm the identity and purity of the sample.

2.5.3 Preparation of *in vitro* run-off transcribed tRNA with specific 3'ends

Plasmid G73 was digested with restriction enzyme *Fok*I (NewEngland Biolabs) to provide a template for run-off transcription which would generate a tRNA ending at the

discriminator base (G73). Digestion of pmBsDCCA with *Fok*I (NewEngland Biolabs), *Bpi*I (Fermentas), or *Bst*OI (Promega), produced transcripts ending at C74, C75, and A76, respectively. Run-off transcription with each linearized DNA template was performed in a total reaction volume of 100 µL containing 20 µL 5X transcription buffer, 5 µL of 10 mM UTP, 5 µL 10 mM CTP, 5 µL 10 mM ATP, 5 µL 1 mM GTP, 50 µCi [α^{32}-P] GTP (10 µCi/µL, 3000 Ci/mmol), 10 µg of linearized DNA template, 60 units of T7 RNA polymerase (Fermentas) and water. The reaction was incubated at 37°C for three hours and terminated with 200 µL phenol, 5 µL 0.5 mM EDTA, 75 µL dH_2O, and 18 µL 3 M NaOAc (pH4.6). The terminated reactions were vortexed and centrifuged at 16 000 xg, 4°C, for 5 minutes. The transcripts were precipitated with two volumes of 99% EtOH and incubated at -70°C for at least an hour. The samples were centrifuged at 16 000 xg, 4°C, for 30 minutes. The supernatant was removed and the pellets were dried in a desiccator for twenty minutes. The pellet was resuspended in 8 µL RNase-free dH_2O and 8 µL Peattie's loading buffer (Table 2-1) (Peattie, 1979). The samples were incubated at 65°C for 10 minutes, cooled on ice and loaded onto a 4 cm by 10 cm 20% polyacrylamide/7 M urea denaturing gel (see section 2.5.4). Electrophoresis was carried out at 200 volts for 3 hours and the gel removed from the apparatus. The surface of the gel was covered with plastic wrap and a piece of X-ray film (Super RX Fujifilm) was placed over the gel for 1½-2 hours of autoradiography. The gel was aligned with the developed film and the transcripts were excised from the gel and transferred to individual 1.5 mL microfuge tubes containing 400 µL of phenol. The gel slices were crushed with a sterile glass rod and 0.5 M NH_4OAc, 10 mM $Mg(OAc)_2$, and1 mM EDTA was added to a final volume of 400 µL. The tubes were rotated overnight at 4°C on the rotisserie and

centrifuged at 16 000 xg, at 4°C, for 5 minutes. The supernatant was transferred to a new microfuge tube and two volumes of 99% EtOH were added. The samples were incubated at -70°C for an hour and the RNA pelleted at 16 000 xg, at 4°C, for 30 minutes. The supernatant was removed and the pellet was resuspended in 400 µL dH_2O containing 0.5 M NH_4OAc by vortexing. The transcripts were EtOH-precipitated once again and the pellet was resuspended in 50 µL of RNase-free H_2O. Transcripts were stored at -20°C.

2.5.4 Denaturing polyacrylamide gels

Each 100 mL polyacrylamide denaturing gel contained 42 g of urea, 11.4 g acrylamide, 0.6 g bis-acrylamide, 650 µL of 10% ammonium persulfate (APS) and 20 µL TEMED for a 12% gel. First, a seal needed to be made at the base of the electrophoresis apparatus. This was performed in one of two ways: forming a plug with 40 mL of 12% 19:1 acrylamide:bisacrylamide, 7 M urea, 120 µL of 20% APS, and 60 µL of TEMED, or by using a gasket supplied by Bio-Rad. The 100 mL of 12% 19:1 polyacrylamide/urea mixture is then inserted into the Bio-RAD apparatus following the accompanying instructions. Wells were made using a 32-well 0.4 mm plastic well-forming comb. Once the gel polymerized, the comb was removed and the gel was submerged in 1X TBE. The wells were rinsed with the 1X TBE buffer prior to loading the samples. The 12% polyacrylamide/urea denaturing gel was electrophoresed for one hour at 700 volts (21 cm x 38 cm) or 1000 volts (50 cm x 38 cm) prior to adding the samples and electrophoresis was carried out at 1600 volts (21 cm x 38 cm) or 1900 volts (50 cm x 38 cm) for 6 hours in 1X TBE.

2.5.5 Activity assays with [α^{32}-P] GTP transcribed tRNA

Labelled transcripts were diluted 10-fold and 100-fold to determine the minimum amount of transcript required to be seen by autoradiography. Standard assay conditions (section 2.5.1) were used for glycine buffer, $MgCl_2$, unlabelled CTP, unlabelled ATP and enzyme but the amount of template was reduced, no radiolabelled nucleotides were added and the reactions were performed in a total volume of 10 µL. Several reaction conditions were tested including both nucleotides, ATP alone, or CTP alone with all other conditions remaining constant. Reactions were terminated by the addition of one volume equivalent of Peattie's loading buffer. The tubes were transferred to boiling water for five seconds and incubated for 10 minutes at 65°C. The reaction mixtures were cooled to room temperature and analyzed after electrophoresis on a 12% denaturing gel. A Geiger counter was used to detect the section of the gel that contained the highest concentration of counts per minute which indicated the position of the run-off transcripts. That section of the gel was excised and transferred to a pick-up film. The Fuji Medical X-ray film (Super RX) was exposed to the gel and incubated at -70°C. The film was developed using a Kodak X-OMAT 1000A film processor.

2.5.6 Time course assay with tRNA-NC

A standard assay mixture was created in a volume of 800 µL containing 100 mM glycine buffer (pH9), 10 mM $MgCl_2$, 0.4 mM CTP, and 1 mM ATP and 800 ng of enzyme. Aliquots (10 µL) were transferred to a microfuge tube containing 10 µL of Peatties loading buffer to terminate the reaction at selected time points: 2, 4, 10, 15, 30, 60, and 120 minutes. The samples were loaded onto a 12% polyacrylamide denaturing

gel and were electrophoresed and processed as mention previously, section 2.5.4 and 2.5.5. This experiment was repeated in the absence of ATP.

2.5.7 Competition assay with radiolabelled tRNA-NC

Reaction mixtures (6 μL) were made the same as for a standard assay (section 2.5.1) except that only one nucleotide was present in concentrations of 1 mM for ATP and 0.4 mM for CTP. After two minutes, 4 μL of the missing nucleotide was added to the reaction. After an additional two minutes, the reactions were terminated with 10 μL of Peattie's loading buffer, boiled for 5 seconds, and incubated for 10 minutes at 65°C. The sample was then analyzed by a 12% polyacrylamide urea denaturing gel (see section 2.5.4).

2.5.8 Competition assay with non-radiolabelled tRNA-NC with [α^{32}P] ATP

Reaction conditions were as for standard reactions (see section 2.5.1) except that the CTP concentrations were varied (0 mM, 0.1 mM, 0.4 mM, and 1 mM). The tRNA substrate was tRNA-NC. Enzyme (100 ng) was used to commence the reaction, and the reaction was terminated with 10 μL Peattie's loading buffer after two minutes. The sample was boiled for 5 seconds and incubated for 10 minutes at 65°C. The sample was then analyzed by a 12% polyacrylamide urea denaturing gel.

3. Results

Transfer RNA nucleotidyltransferase is required to add -C74, -C75, and -A76 when they are absent from the 3' ends of tRNAs. This CCA sequence is required for aminoacylation and, therefore, protein synthesis. This CCA addition is a unique process whereby the sequence is added in a specific but nucleic acid template-independent manner. In this study, attempts were made to assign a role to a conserved arginine residue in tRNA nucleotidyltransferase (Arg244 in the *C. glabrata* enzyme). Mutational analyses in combination with biophysical and biochemical characterization have been employed to better understand the importance and the role of this particular residue.

3.1 Generation of an R244K variant

Previous work included the construction of expression plasmids whereby the *C. glabrata CCA1* gene, as well as mutant *C. glabrata CCA1* genes coding for alanine and methionine variants, were successfully inserted into pGEX-2T. To obtain the lysine variant, site-directed mutagenesis was performed on the *C. glabrata CCA1* gene. The introduction of a *Cfr*9I recognition sequence by the addition of a silent mutation within the newly synthesized DNA facilitated the task of differentiating the mutant from the native gene. After linearizing each plasmid with *Sal*I, each was digested with *Cfr*9I. If the new restriction site had been introduced this should release a 911 bp fragment from the plasmid. As expected, if mutagenesis was successful, this 911 bp fragment was seen (Fig. 3-1, lane 1). In contrast the original plasmid (6585 bp) showed no fragment of this size (Fig. 3-1, lane 2).

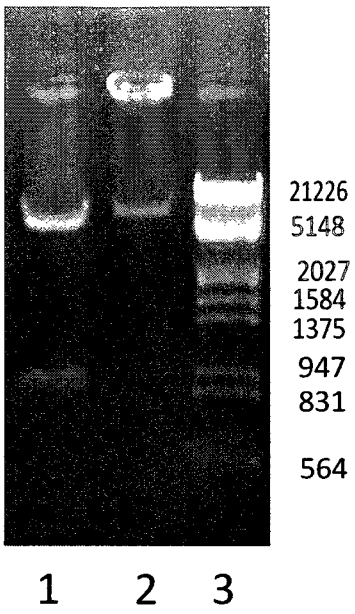


Fig. 3-1. Agarose gel electrophoresis of *Sal*I and *Cfr*9I double-digested plasmids. Lane 1, lysine variant *cca1* plasmid, lane 2, native *CCA1* control plasmid, lane 3, *Eco*RI-*Hin*dIII digested lambda marker DNA. The sizes of the fragments in base pairs (bp) for the lambda marker are shown.

3.2 Expression and purification of native and variant tRNA nucleotidyltransferases

After transforming plasmids coding for the native and variant tRNA nucleotidyltransferases (R244A, R244K, R244M) into *E. coli*, the recombinant proteins were overexpressed and purified as determined by SDS-PAGE (Fig. 3-2). The predicted size of the GST-tRNA nucleotidyltransferase fusion protein was ~90kDa, (as seen in the crude lysate Fig. 3-2, lane 2), while the tRNA nucleotidyltransferase alone was ~60kDa (purified proteins Fig. 3-2, lanes 7, 8, 9, 10). Since thrombin cleavage occurs on the column, the 26 kDa GST-tag remains bound to the glutathione resin allowing the 60 kDa *CCA1* enzyme to elute. The native enzyme and R244 variants did not show any major difference in expression levels.

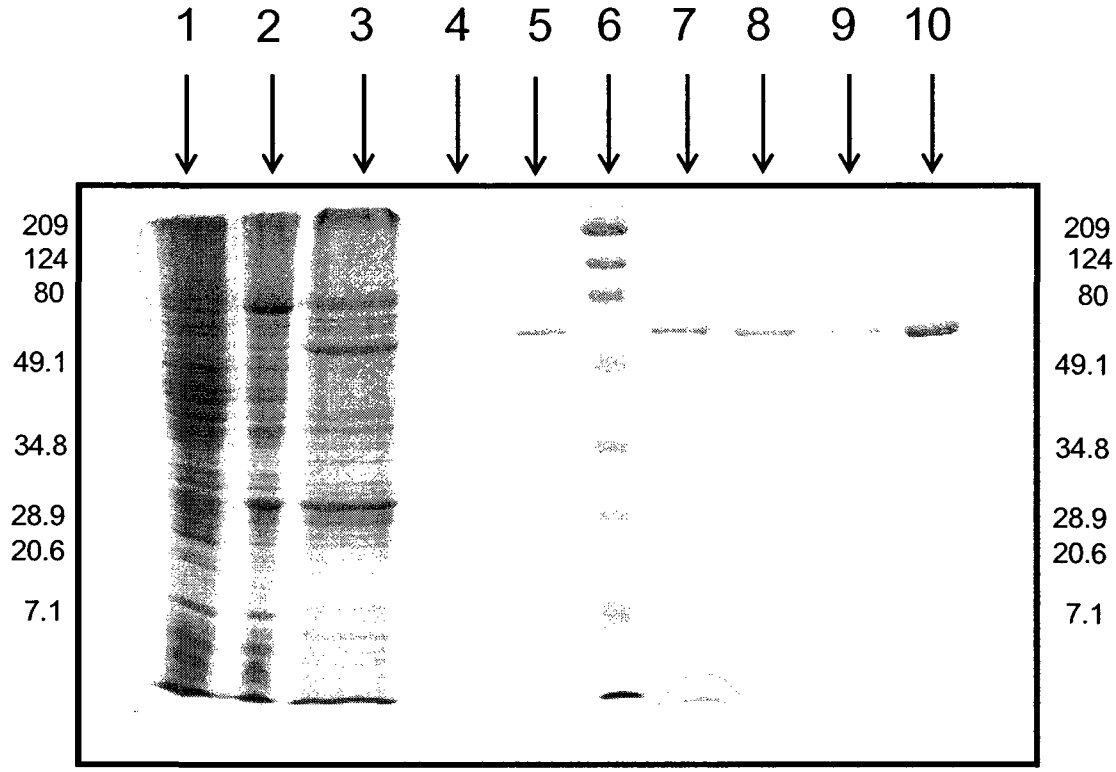


Fig. 3-2. Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel representing the stepwise purification of native and variant forms of tRNA nucleotidyltransferase expressed from pGEX-2T. Lane 1: crude cell lysate (native enzyme) non-induced; lane 2: crude cell lysate (native enzyme) induced with 1 mM IPTG; lane 3: flow through from glutathione column; lane 4: after 2X PBS wash; lane 5: after thrombin cleavage; lane 6: Bio-rad pre-stained SDS-PAGE standards, broad range; lane 7: alanine variant (1 μg); lane 8: lysine variant (1 μg); lane 9: methionine variant (1 μg); lane 10: native enzyme (3 μg). Sizes of molecular weight markers (kDa) are shown to the left and right of the gel.

3.3 Biophysical characterization of tRNA nucleotidyltransferase

3.3.1 Determination of secondary structure by circular dichroism

To evaluate the effects of the point mutations altering amino acid 244 on the secondary structure of the protein, each protein sample was scanned in the far-UV region, between 200 nm and 260 nm, with a J-815 circular dichroism system. The spectra of the native and variant enzymes are shown separately (Fig. 3-3) and overlaid (Fig. 3-4). They all show typical circular dichroism spectra for α-helical structures with expected minima

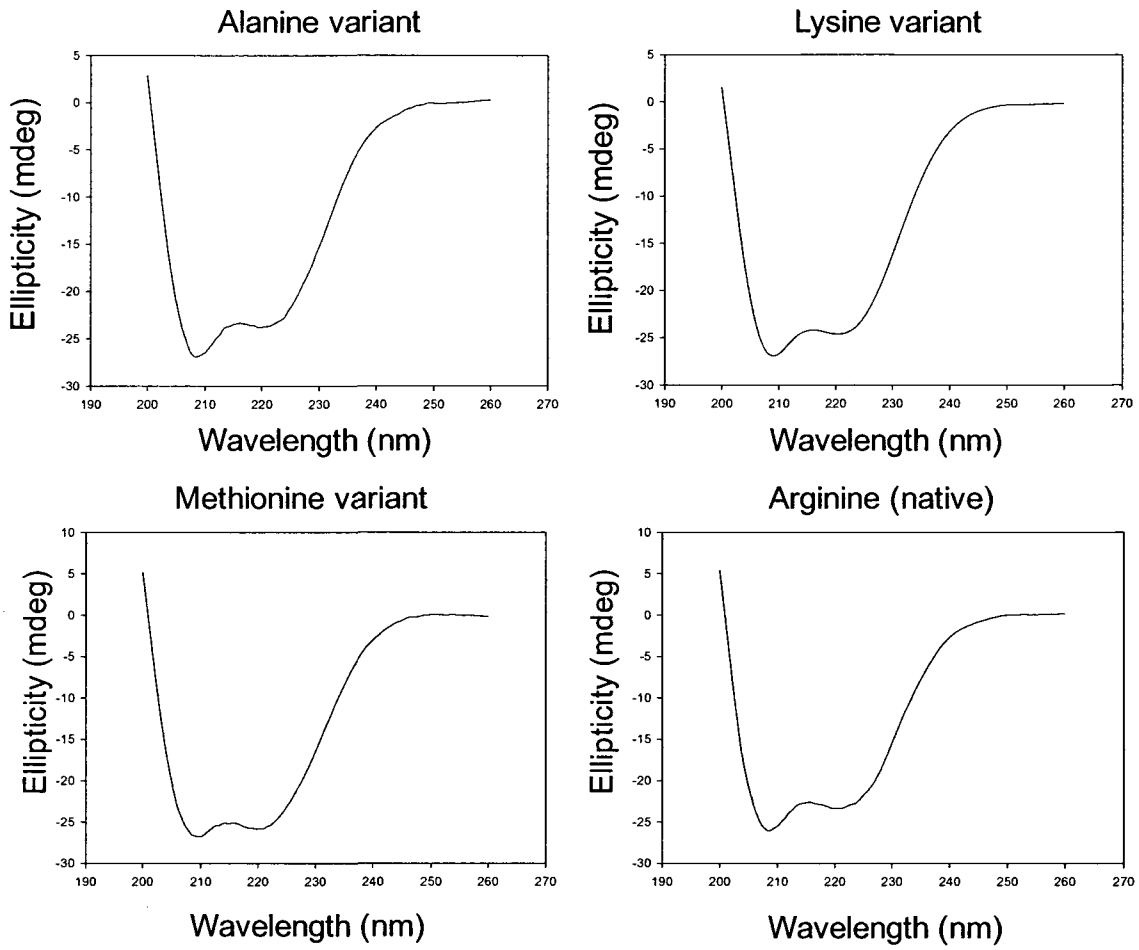


Fig. 3-3. Far-UV circular dichroism spectra of native and variant tRNA nucleotidyltransferases. Circular dichroism spectra were generated in the far-UV region between 200 nm and 260 nm with 200 µL of 0.262 mg/mL of protein in a 0.1 cm quartz cuvette in 1X PBS (pH 7.4).

at 208 nm and 222 nm. This is consistent with what has been seen in numerous tRNA nucleotidyltransferase crystal structures (Li *et al.*, 2002; Augustin *et al.*, 2003; Tomita *et al.*, 2004) which show primarily α-helices. Despite small variations in ellipticity, the spectra show that the secondary structure is highly similar between the native enzyme and the alanine and lysine variants. The methionine variant demonstrates a stronger signal for α-helical secondary structure at 222 nm and demonstrates a slight variability in shape versus the other three enzymes. All four enzymes demonstrate an intensity peak of

-26 mdeg at 208 nm and of -24 mdeg ±1 mdeg at 222 nm indicating that changing arginine244 to alanine, lysine, or methionine has no major effect on the secondary structure of *C. glabrata* tRNA nucleotidyltransferase.

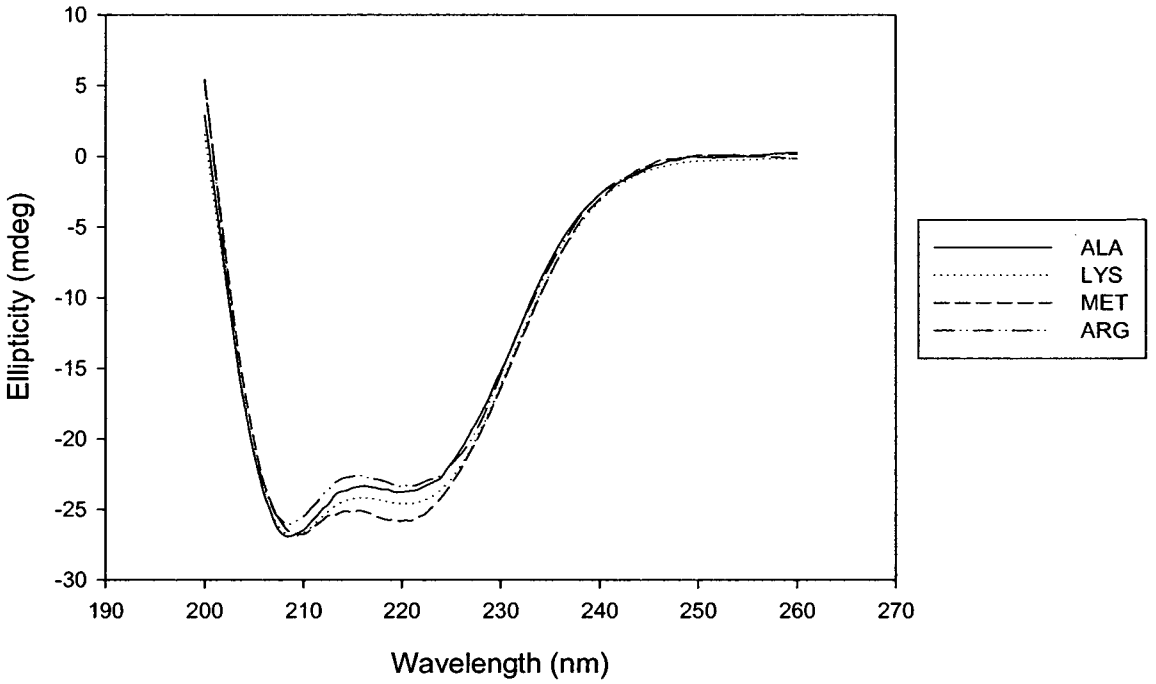


Fig. 3-4. Overlaid Far-UV CD spectra of native and variant tRNA nucleotidyltransferases (see Fig. 3-3).

3.3.2 Temperature-induced denaturation of native and variant enzymes detected by circular dichroism spectroscopy

Yeast cells transformed with plasmids containing one of the three possible variants (R244A, R244K, or R244M) could not survive on synthetic complete medium at 30°C (Fig. 1-8). The demonstrated phenotype can be engendered by either an unstable or inactive tRNA nucleotidyltransferase, resulting in the loss of protein synthesis. To determine whether or not the mutations at this position led to a decrease in the stability of the enzyme, temperature denaturation studies were performed by circular dichroism spectroscopy. The temperature scan was performed from 20°C to 95°C (Fig. 3-5). The

melting temperatures were determined to be 38.4°C, 39.2°C, 41.8°C and 42.6°C for arginine, lysine, methionine, and alanine enzymes, respectively (Table 3-1). The shapes of all of the curves demonstrate a cooperative two-state unfolding for all four enzymes indicating that the changes at this position did not alter the overall process of unfolding in this enzyme (Creighton, 1989). Slight ellipticity differences seen at the beginning

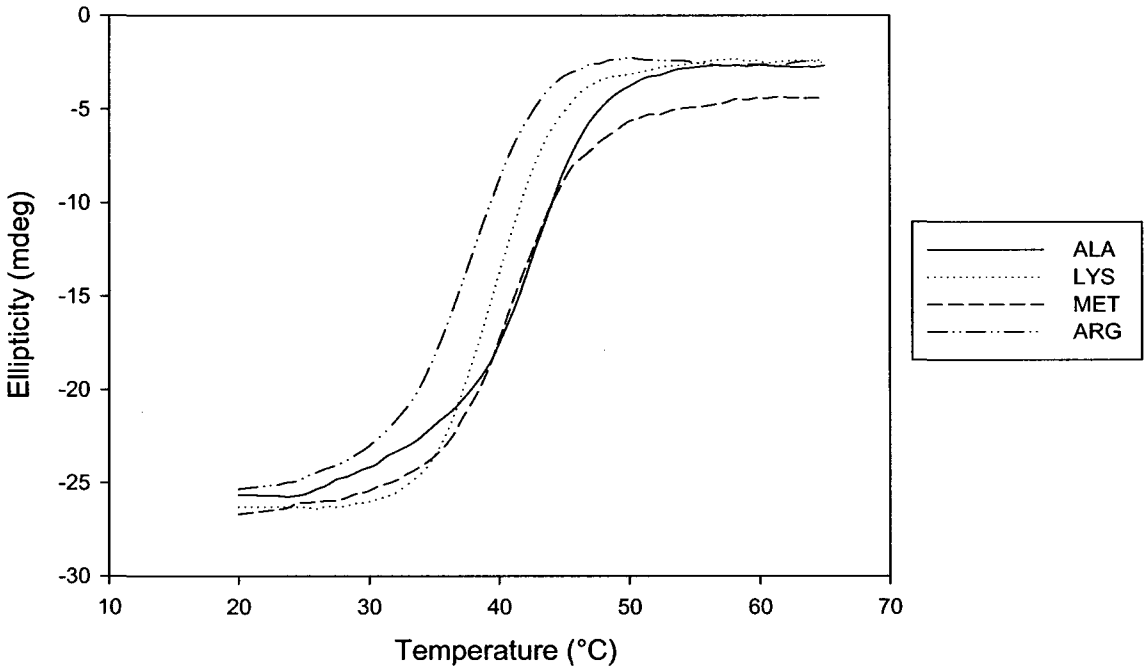


Fig. 3-5. Temperature-induced denaturation of native and variant enzymes detected by circular dichroism spectroscopy. Signal intensity at 208 nm is shown for temperatures between 20°C and 65°C for all four enzymes.

of the measurement stem from small variations in enzyme concentration, whereas those found at the end of the measurement indicate enzyme precipitation when completely denatured.

Enzyme	Tm	Tm variant -Tm native
ARG	38.4°C	-
LYS	39.2°C	0.8°C
MET	41.8°C	3.4°C
ALA	42.6°C	4.2°C

Table 3-1. Melting temperatures of *C. glabrata* tRNA nucleotidyltransferase. Melting temperatures determined by taking the first derivative of the ellipticity values using the J-815 SpectraAnalysis software.

3.3.3 Characterization of tRNA nucleotidyltransferase tertiary structure as determined by fluorescence spectroscopy

To determine whether or not the tertiary structure of the protein was affected by the amino acid changes at position 244, fluorescence spectroscopy was employed to assess any shifts in the environment of the aromatic residues of the enzymes. Fluorescence spectroscopy relies on the excitation of the aromatic residues phenylalanine, tyrosine, and tryptophan. Once these residues have absorbed electrons at a specific wavelength, they emit at longer wavelengths. The emission spectrum depends on the polarity of the environment within which the residues lie. The emission spectrum also depends on the proximity of tyrosine residues to tryptophan residues since the latter quenches the fluorescence of the former (Creighton, 1989). The emission spectra of all proteins excited at 280 nm revealed a peak maximum around 329 nm (Table 3-2). There is no major shift due to the changes at position 244 of tRNA nucleotidyltransferase (Fig. 3-6 and Fig. 3-7).

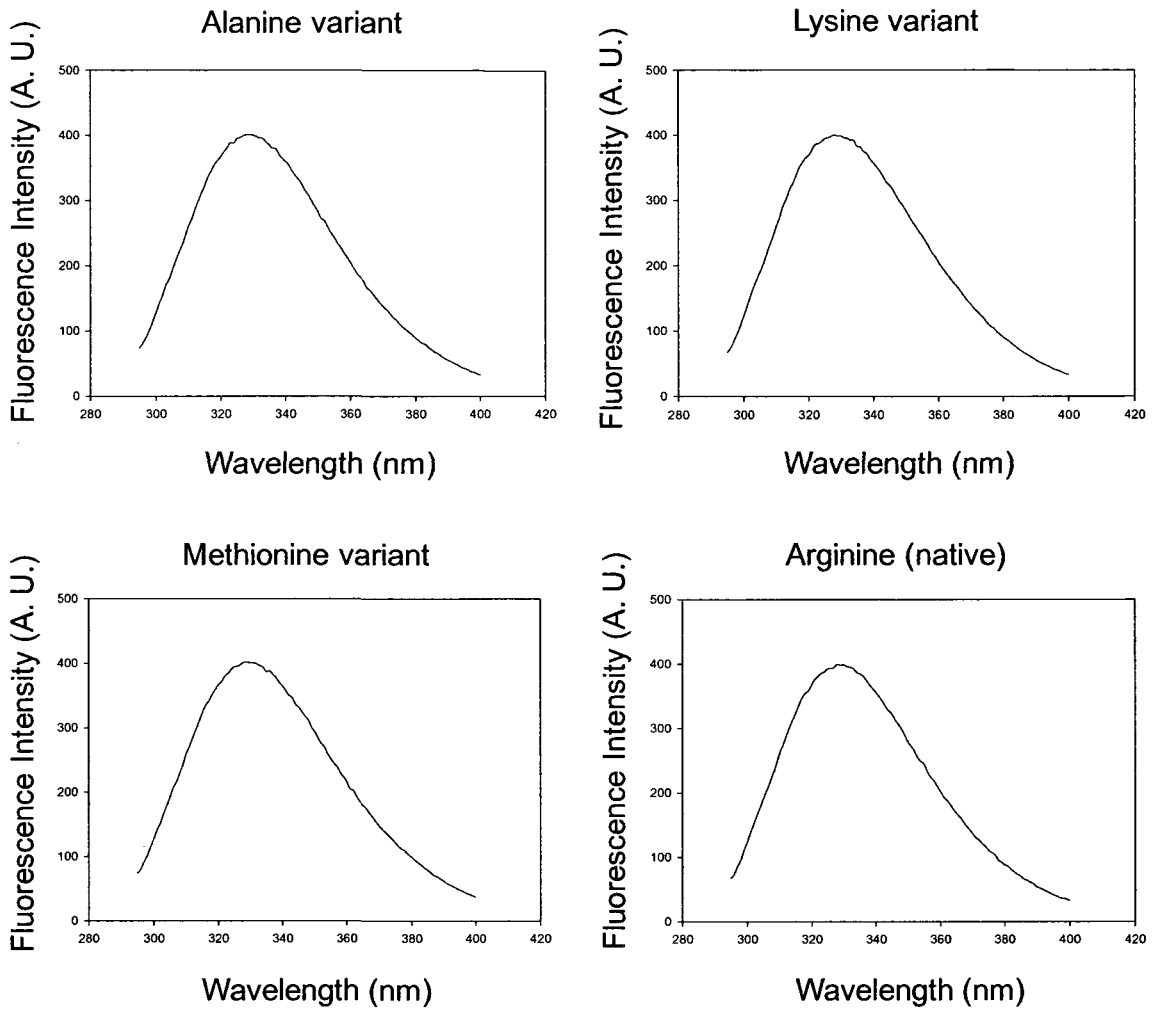


Fig. 3-6. Fluorescence emission spectra of native and variant tRNA nucleotidyltransferases. Samples were excited at 280 nm and fluorescence emission was observed between 295 nm and 400 nm in a 10 mm fluorescence quartz cuvette.

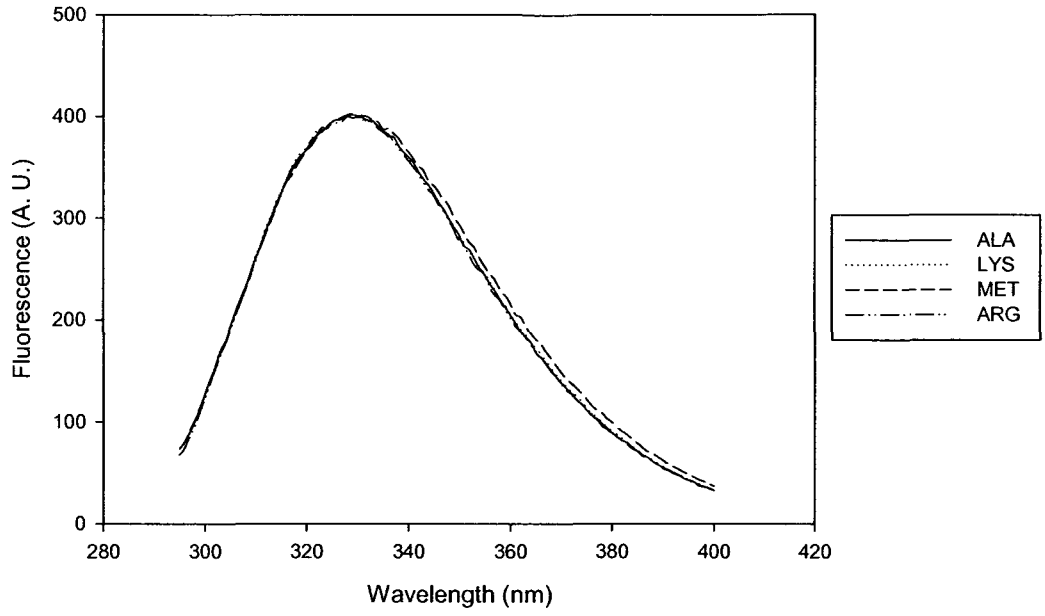


Fig. 3-7. Overlaid fluorescence emission spectra of native and variant tRNA nucleotidyltransferases excited at 280 nm (see Fig. 3-6).

Enzyme	λ max (nm)
Alanine	329
Lysine	328
Methionine	329
Wild-type	330

Table 3-2. Peak intensity of arginine and variant tRNA nucleotidyltransferases observed by fluorescence spectroscopy.

3.4 Enzyme assays

3.4.1 Acid precipitable counts

Since no change in the stability or structure of the enzyme was seen, standard activity assays were performed to see if the loss of viability resulted from a loss of enzyme activity. Preliminary characterization of Arg244 and the Ala and Met variants using the standard assay conditions developed for the *S. cerevisiae* enzyme (Shan, 2005;

Shan *et al.*, 2008) demonstrated that the variants had 54.8% (Ala) and 62.4% (Met) activity relative to Arg244 (Fig. 3-8). The lysine variant was unavailable at the time this initial assay was performed and was omitted. Based on these results, it did not seem that the variants lost a sufficient amount of activity to explain the observed *in vivo* phenotype (Fig. 1-8). To further explore the activity of the enzyme the same assay was carried out but with labelled CTP instead of ATP. The results of these assays were much more dramatic as the variants demonstrated no activity above background (boiled enzyme control). This suggested a number of possibilities, for example, the variants may have

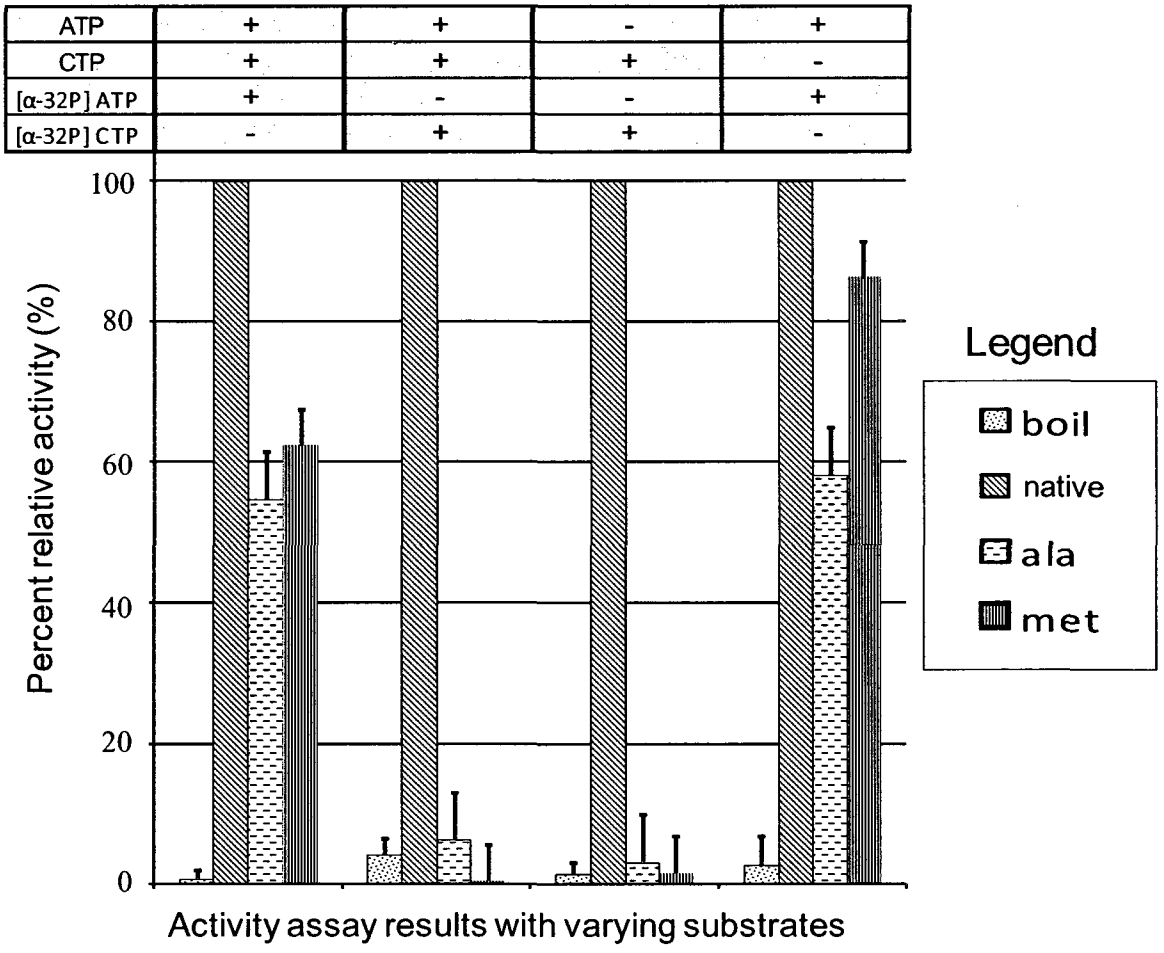


Fig. 3-8. Summary of enzyme activity as measured by acid precipitable counts. Variant activity was calculated relative to Arg244.

lost the ability to discriminate between CTP and ATP (*i.e.*, ATP was being incorporated preferentially at positions 74, 75, and 76), the ability to incorporate CTP at position 74, position 75, or both positions was severely impaired, the crude cellular tRNA population contained only a limited number of tRNAs lacking the C residues at positions 74 and 75 (such that the original assay is only measuring the incorporation of A76), or some combination of these. To distinguish between these possibilities, the assays were repeated with one of the nucleoside triphosphates removed from the reaction mix. When ATP was removed and the assay carried out with only CTP, the activity levels for both variants were, again, similar to those found for the boiled enzyme indicating that the loss in activity was not simply due to misincorporation of ATP instead of CTP at positions 74 and 75. When the CTP was removed completely and the assay was performed in the presence of only ATP, the alanine variant showed the same level of activity as it did in the standard assay (58.2%) suggesting that CTP incorporation makes up only a small fraction of the reaction, *i.e.*, most of the substrate tRNA molecules already contain C residues at positions 74 and 75. In contrast, an increase in activity (64.2% with CTP up to 86.2% without CTP) was seen with the methionine variant. One possible explanation for this observation is that in the presence of CTP the methionine variant may misincorporate it in place of A more so than do the native or alanine variants. It is interesting that the Ala and Met variants show differences both in their CD spectra (Fig. 3-3, 3-4) and in terms of the incorporation of acid precipitable counts suggesting that while both of these variants effect the activity of the enzyme they may do so in slightly different ways. In general terms, these data suggest that while the variant enzymes (Ala and Met) are still able to incorporate labelled nucleotides into the tRNAs at fairly high levels relative to the

native enzyme they seem to have greater difficulty either in recognizing or incorporating CTP.

It is important to note that even the native enzyme showed a variation in activity depending on the labelled nucleotide used. When calculated as the absolute number of counts incorporated (see appendix) the native enzyme showed twice as many counts incorporated from radioactive ATP as compared to radioactive CTP in the standard assay suggesting that most of the tRNA in the crude yeast tRNA preparation already contained an intact C74 or C75. This suggestion is further supported by the fact that if the tRNAs lacked positions 74, 75, and 76 one would expect twice as much CTP to be incorporated as ATP. If only C74 remained, the levels of incorporation of each nucleotide would be equivalent since only one of each nucleotide would have been added.

3.4.2 Run-off transcription assays

3.4.2.1 Restriction enzyme digests of G73 and pmBsDCCA plasmids

To more precisely define the effect of the changes at position 244 on the addition of ATP or CTP specifically, plasmids with the gene encoding *B. subtilus* Asp-tRNA^{GUC} were used to make tRNA substrates with specific 3' termini. Plasmids G73 and pmBsDCCA were digested with specific enzymes to generate *in vitro* run-off transcription products ending at the positions indicated (Table 3-3). 'N' represents the discriminator base at position 73 which is a guanosine in *B. subtilus* Asp-tRNA^{GUC}.

Plasmid	Restriction enzyme	3'-terminus
G73	*Fok*I	tRNA-N(73)
pmBsDCCA	*Fok*I	tRNA-NC(74)
pmBsDCCA	*Bpi*I	tRNA-NCC(75)
pmBsDCCA	*Bst*OI	tRNA-NCCA(76)

Table 3-3. Producing tRNAs with specific 3' ends. Left: plasmids containing the gene coding for *B. subtilus* Asp-tRNA^{GUC}; centre: restriction enzyme used to linearize plasmid; right: tRNA product obtained after *in vitro* run-off transcription.

Agarose gel electrophoresis was used to show that restriction digestion had reached completion. The expected fragment sizes following complete digestion are: G73 and pmBsDCCA with *Fok*I: ~1331, 643, 287, 244, and 181 bp; pmBsDCCA with *Bpi*I: ~2686 bp; and pmBsDCCA with *Bst*OI: ~2071, 288, 191, 123, and 13 bp (Fig. 3-9).

3.4.2.2 Run-off transcription

When radiolabelled [α-^{32}P]-GTP was used in the transcription assay a total of ~70% of the label was incorporated into the transcripts. The transcripts were then separated by denaturing polyacrylamide gel electrophoresis, excised, and approximately ~90% of the transcripts were recovered from the gel (Fig 3-10).

3.4.2.3 Transfer RNA nucleotidyltransferase assays with run-off transcripts

After run-off transcription of the digested plasmids, the first radiolabelled transcript to be tested was the tRNA ending at the discriminator base and lacking CCA. When this transcript was used as a substrate under standard conditions the Arg244 enzyme efficiently added nucleotides to positions 74, 75, and 76 (Fig. 3-11, Table 3-4). When Arg244 was supplied with only ATP it could add a single AMP to position 74. This improper A addition under *in vitro* conditions has previously been seen with several other tRNA nucleotidyltransferases (Deutscher, 1983). Under conditions where CTP was

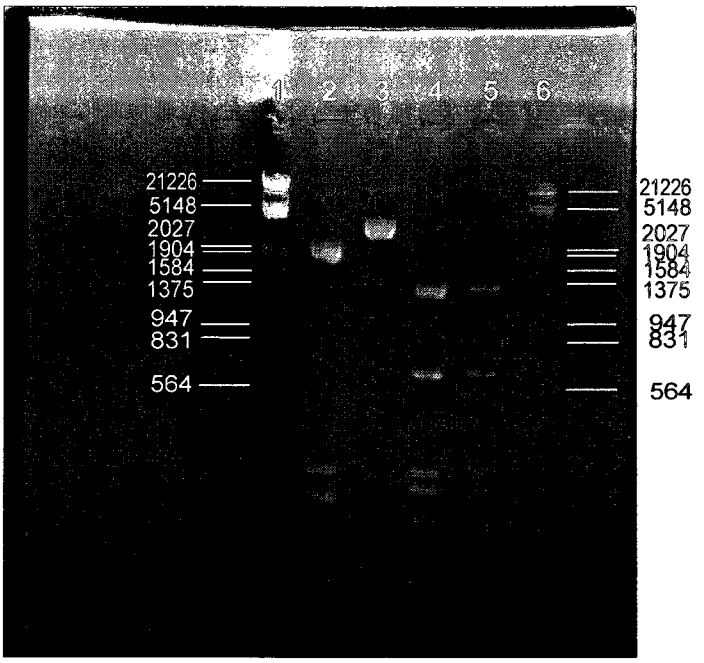


Fig. 3-9. Digested G73 and pmBsDCCA plasmids analyzed by agarose gel electrophoresis. Lanes 1 and 6: *Eco*RI and *Hin*dIII digested lambda DNA ladder (Fermentas); lanes 2-4: *Bst*OI, *Bpi*I, and *Fok*I digested pmBsDCCA plasmid, respectively; lane 5: *Fok*I digested G73 plasmid.

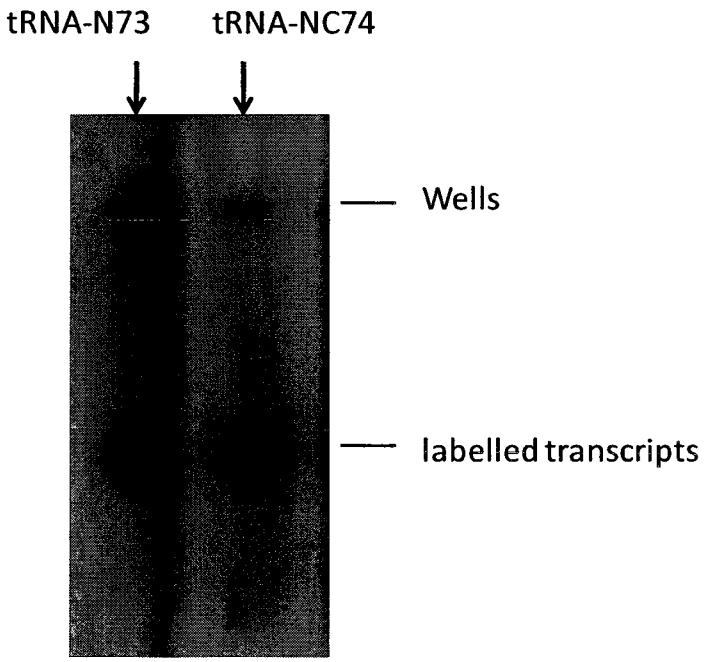


Fig. 3-10. Autoradiogram of run-off transcription products separated by 20% polyacrylamide urea denaturing gel.

the only nucleotide present, Arg244 added three cytidines. This has also been observed previously under *in vitro* conditions for the *E. coli* and rabbit liver enzymes (Deutscher, 1973a; Deutscher, 1973b; Hou, 2000; Seth *et al.*, 2002). In stark contrast to this, the variants could not add either nucleotide at position 74 to extend the tRNA at all (Fig. 3-11, Table 3-4). This immediately suggests why these enzymes cannot support life *in vivo*, as primary transcripts ending at the discriminator base may not be extended efficiently. A faint band suggests the possibility that the lysine variant may be able to add at this position inefficiently (Fig. 3-12c, lane 1), but cannot extend this to a complete CCA terminus. This may imply that the arginine residue plays a vital role in either CTP binding or catalysis at position 74.

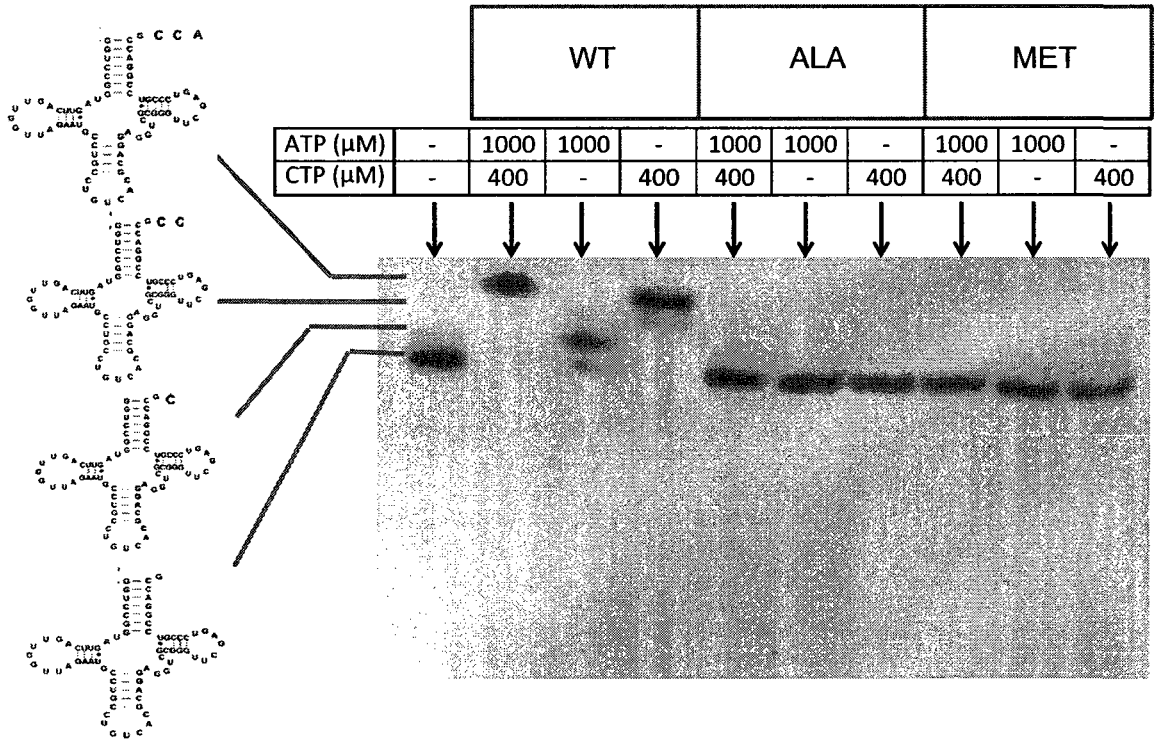


Fig. 3-11. Products from standard or modified assay conditions utilizing the substrate tRNA-N and analyzed on a 12% denaturing gel. Lane 1, tRNA-N no enzyme; Asp-tRNA^{GUC} from *B. subtilus* is shown on the left.

	Native			Alanine			Methionine		
	ATP/CTP	ATP	CTP	ATP/CTP	ATP	CTP	ATP/CTP	ATP	CTP
NCCA	100	-	94.7	-	-	-	-	-	-
NCC	-	3.6	5.3	-	-	-	-	-	-
NC	-	84.8	-	-	-	-	-	-	-
N	-	11.6	-	100	100	100	100	100	100

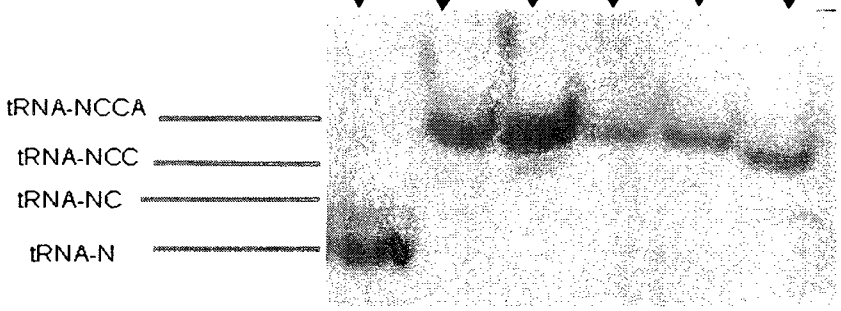
Table 3-4. Percentage of resulting tRNAs obtained with tRNA-N in standard or modified conditions. Percentage of each product in a single lane is shown for the native, alanine, and methionine enzymes for the substrate tRNA-N in standard assay conditions (ATP/CTP), in the presence of ATP alone (ATP), and in the presence of CTP alone (CTP). Percentage calculated by densitometry using GeneTools (Syngene).

3.4.2.4 Standard tRNA nucleotidyltransferase reaction conditions with tRNA-N, -NC, -NCC, and -NCCA

To further characterize the potential defects in the variant enzymes their activity was tested with the other possible tRNA substrates (-NC, -NCC, and -NCCA). The native enzyme (Arg244) always extended the 3' end of the tRNA substrates to include position 76 under standard assay conditions (Fig. 3-12a). As shown previously, the alanine and methionine variants did not add efficiently at the discriminator base but if provided with a substrate containing CMP already at this position, the variants can extend this inefficiently whereby bands are seen at equivalent sizes to tRNA-NCC and tRNA-NCCA (Fig. 3-12b, c, d), further suggesting an impairment in CTP incorporation particularly at position 74. When the variants were provided with tRNA-NCC, all three were able to add at position 76 (Fig. 3-12b, c, d). A general theme appears from these results whereby the variants have a greater difficulty in CTP addition as compared to ATP at all positions tested.

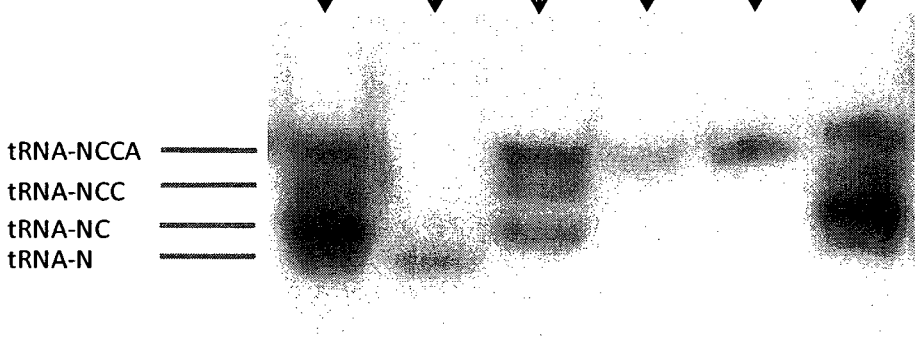
a)

Transcript	N	N	NC	NCC	NCCA	NCCA
Enzyme	-	+	+	+	+	-



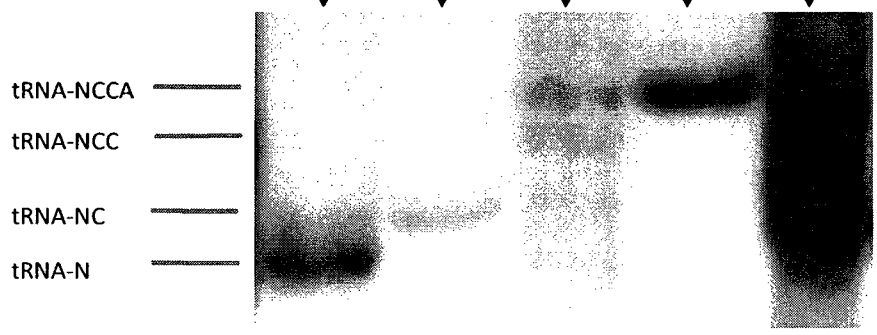
b)

Transcript	T4	N	NC	NCC	NCCA	T4
Enzyme	-	+	+	+	+	-



c)

Transcript	N	NC	NCC	NCCA	T4
Enzyme	+	+	+	+	-



d)

Transcript	T4	N	NC	NCC	NCCA	T4
Enzyme	-	+	+	+	+	-

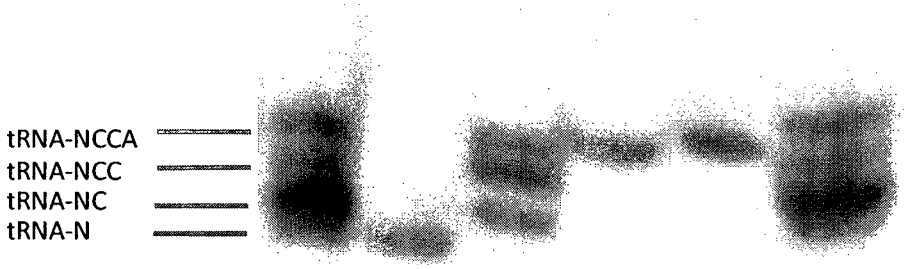


Fig. 3.12. Assays containing transcribed tRNAs in standard conditions. a) native enzyme, b) alanine variant, c) lysine variant, d) methionine variant. The transcript present in each reaction is shown in the table above each lane and the presence (+) and absence (-) of the enzyme is also indicated. A mixture of all four transcripts are marked as T4 and included as a control. The terminated reactions are analyzed by 12% denaturing gel.

a)

	Substrate			
	N	NC	NCC	NCCA
NCCA	100	100	100	100
NCC	-	-	-	-
NC	-	-	-	-
N	-	-	-	-

b)

	Substrate			
	N	NC	NCC	NCCA
NCCA	-	35	100	100
NCC	-	12.5	-	-
NC	-	52.5	-	-
N	100	-	-	-

c)

	Substrate			
	N	NC	NCC	NCCA
NCCA	-	-	97.9	100
NCC	-	-	2.1	-
NC	-	100	-	-
N	100	-	-	-

d)

	Substrate			
	N	NC	NCC	NCCA
NCCA	-	15.4	100	100
NCC	-	38.8	-	-
NC	-	45.8	-	-
N	100	-	-	-

Table 3-5. Percentage of resulting tRNAs obtained from standard assay conditions with transcribed tRNAs. a) native enzyme, b) alanine variant, c) lysine variant, d) methionine variant. Percentage of each product formed for each respective substrate is calculated by densitometry using GeneTools (Syngene).

3.4.2.5 Time course with tRNA nucleotidyltransferase variants

Since little of the tRNA-NC template was fully extended under the standard enzyme assay conditions, a time course was performed to see if a longer incubation time would result in a larger amount of the products being extended. All three variant enzymes were incubated under standard conditions with tRNA-NC over a span of two hours, with 10 μL aliquots removed at specific time points and transferred to 10 μL of Peattie's sample buffer to terminate the reaction. All of the variants showed incorporation of at least one nucleotide into the substrate within four minutes, but none showed complete extension over the two hour time course of the experiment (Fig. 3-13). In fact, extension of the tRNA to the third position occurred for 70%, 98%, and 35% (Table-3-6) of the total tRNA for alanine (Fig. 3-13a), lysine (Fig. 3-13b), and methionine (Fig. 3-13c) variants, respectively. The results from the alanine and methionine variants may imply one of three things: that the enzymes add CTP inefficiently, that CTP is not incorporated and ATP is incorporated inefficiently in place of CTP, or that competition occurs between CTP and ATP such that once an ATP is added no further extension can occur. Further experiments are required to address these possibilities.

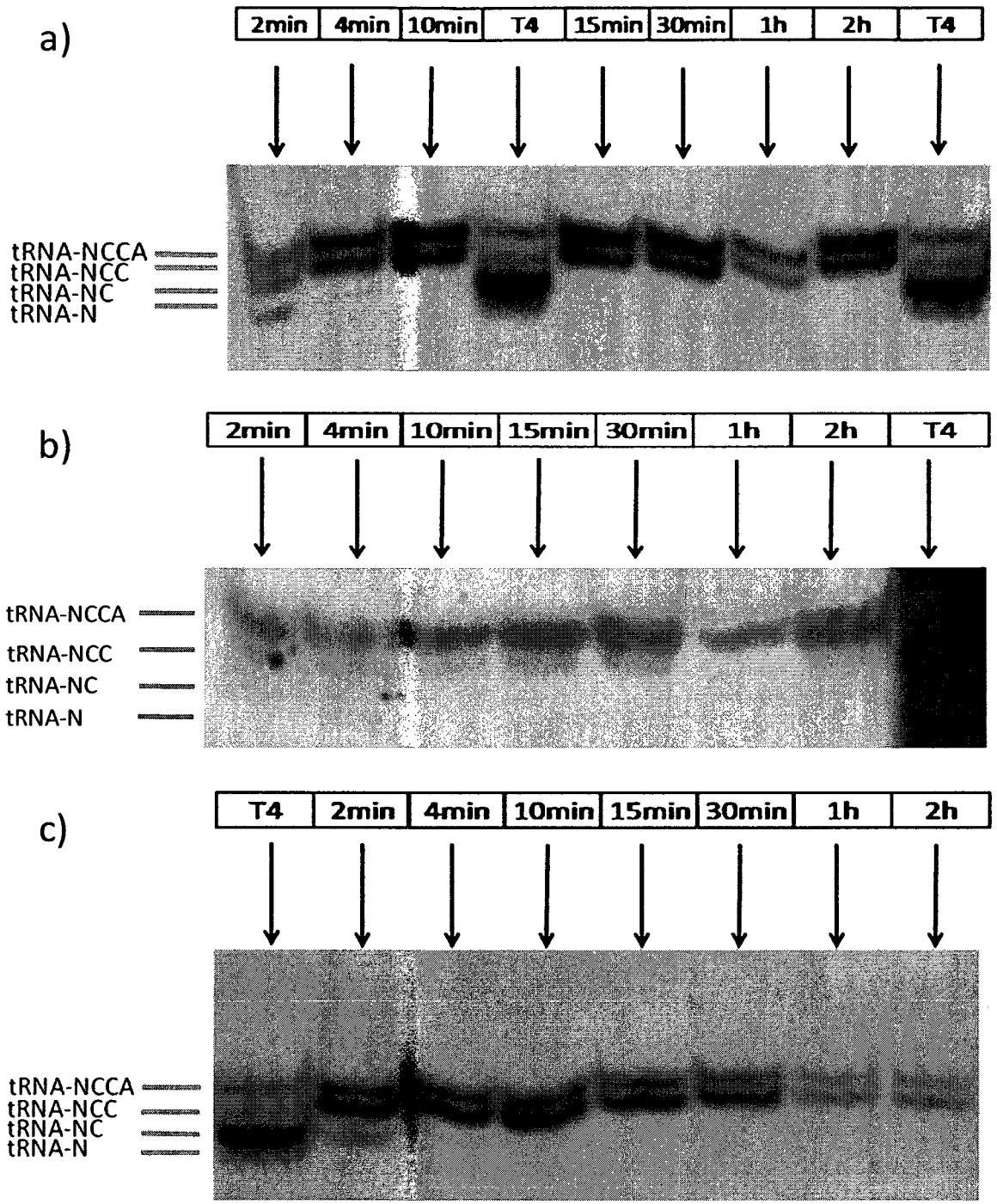


Fig. 3-13. Time course with tRNA-NC in standard conditions. Transfer RNA-NC incubated with ATP and CTP in the presence of a) alanine variant b) lysine variant c) methionine variant. Samples of the reaction were removed and terminated at the times indicated. A mixture of all four transcripts are marked as T4 and included as a control. Terminated reactions are analyzed by 12% denaturing gel.

a)

	Length of Reaction (minutes)						
	2	4	10	15	30	60	120
NCCA	45.1	59.5	65.9	73.8	76.8	63.7	68.4
NCC	16.4	29.9	34.1	26.2	23.2	36.3	31.6
NC	38.5	10.6	-	-	-	-	-

b)

	Length of Reaction (minutes)						
	2	4	10	15	30	60	120
NCCA	88.1	99.3	97.5	95.2	97.4	96.3	98.1
NCC	11.9	0.71	2.5	4.8	2.6	3.7	1.9
NC	-	-	-	-	-	-	-

c)

	Length of Reaction (minutes)						
	2	4	10	15	30	60	120
NCCA	30.4	41.3	36.8	37.1	37.6	23.6	23.1
NCC	50.5	58.7	63.2	62.9	62.4	76.4	76.9
NC	19.1	-	-	-	-	-	-

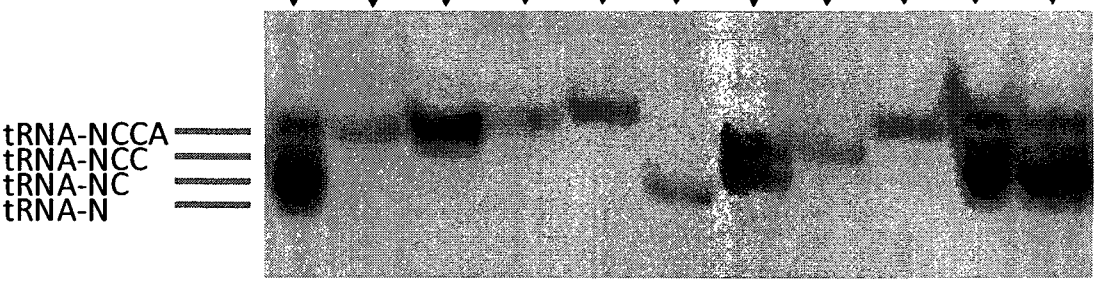
Table 3-6. Percentage of resulting tRNAs from a time course with tRNA-NC in standard conditions. a) alanine variant, b) lysine variant, c) methionine variant. Percentage of each product formed after 2, 4, 10, 15, 30, 60, and 120 minutes with tRNA-NC as the substrate. Percentage of each band in each lane is calculated by densitometry using GeneTools (Syngene).

3.4.2.6 Enzyme assays with run-off transcripts in the absence of ATP.

To distinguish between inefficient CTP addition or ATP incorporation blocking elongation from position 74, the standard reaction was repeated in the absence of ATP. As seen previously, the native enzyme added one or multiple CTPs depending on the transcript given and always resulted in tRNA with -C-C-C endings (Fig. 3-14a). In contrast, the alanine and methionine variants did not add C74 to tRNA-N, extended tRNA-NC to tRNA-NCC, and did not generate tRNA-NCCC (Fig. 3-14a and 3-14c). Of particular interest, the lysine variant did not extend tRNA-N or tRNA-NC, but efficiently added C to tRNA-NCC to generate tRNA-NCCC (Fig. 3-14b). This indicated that the variants recognized CTP but that CTP addition was inefficient.

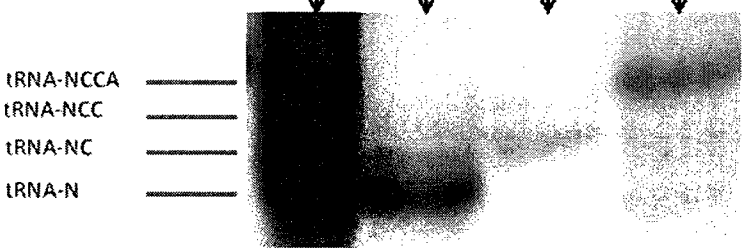
a)

Transcript	T4	N	NC	NCC	NCCA	N	NC	NCC	NCCA	T4	T4
Enzyme	-	WT	WT	WT	WT	ALA	ALA	ALA	ALA	-	-



b)

Transcript	T4	N	NC	NCC
Enzyme	-	LYS	LYS	LYS



c)

Transcript	N	-	N	NC	NCC	NCCA	NCCA
Enzyme	-	-	MET	MET	MET	MET	-

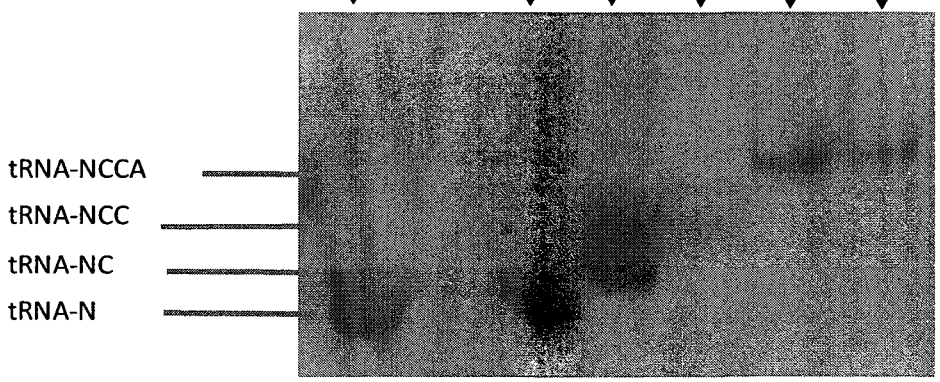


Fig. 3.14. Assays containing transcribed tRNAs in the absence of ATP. a) native enzyme and alanine variant, b) lysine variant, c) methionine variant. Reactions are performed in standard conditions in the absence of ATP. A mixture of all four transcripts are marked as T4 and included as a control. Terminated reactions are analyzed by 12% denaturing gel.

a)

	Substrate							
	Native				Alanine			
	N	NC	NCC	NCCA	N	NC	NCC	NCCA
NCCA	100	100	100	100	-	-	-	100
NCC	-	-	-	-	-	48.2	100	-
NC	-	-	-	-	-	51.8	-	-
N	-	-	-	-	100	-	-	-

b)

	Substrate		
	N	NC	NCC
NCCA	-	-	100
NCC	-	4.4	-
NC	8.3	95.6	-
N	91.7	-	-

c)

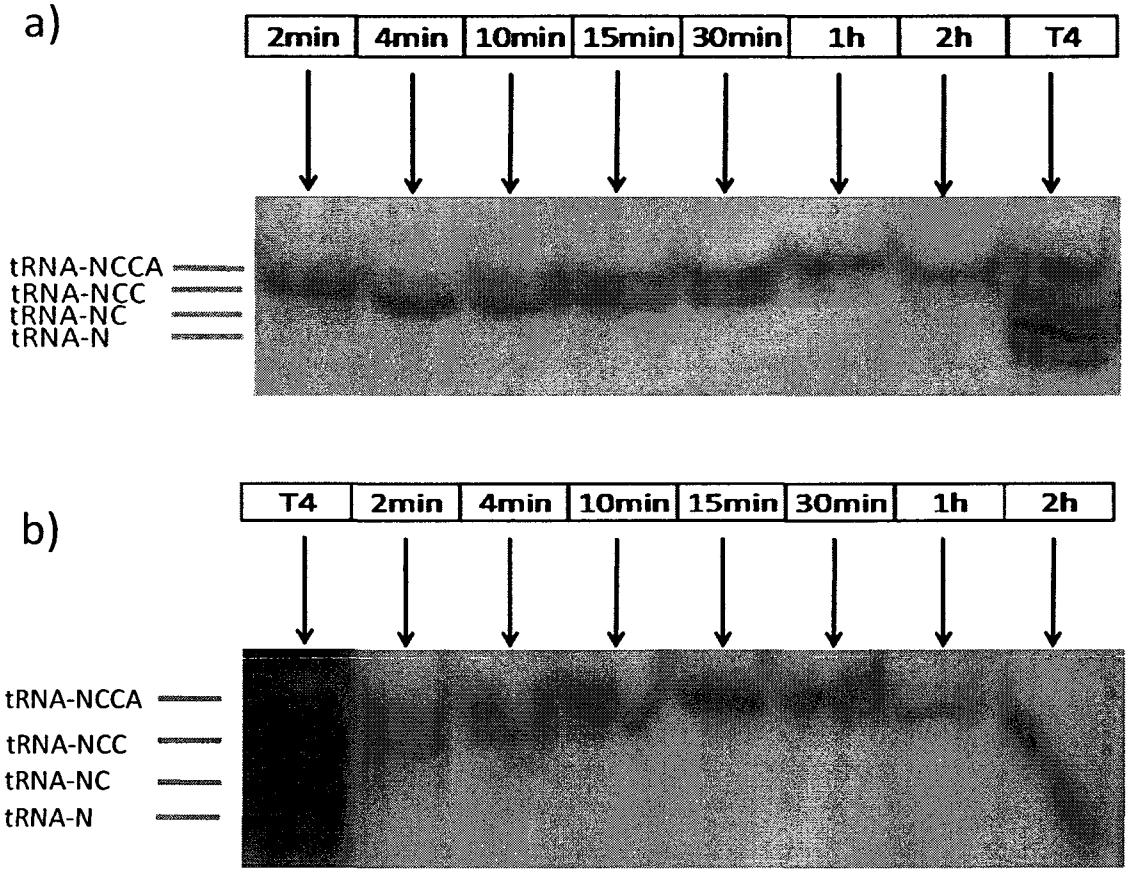
	Substrate			
	N	NC	NCC	NCCA
NCCA	-	-	-	100
NCC	-	36.4	100	-
NC	-	63.6	-	-
N	100	-	-	-

Table 3-7. Percentage of resulting tRNAs obtained from assays containing transcribed tRNAs in the absence of ATP. a) native enzyme and alanine variant, b) lysine variant, c) methionine variant. Percentage of each product formed is calculated by densitometry using GeneTools (Syngene).

3.4.2.7 Time course with variants with tRNA-NC in the absence of ATP

Once it was established that CTP could be recognized by the variants, the time course assay was repeated in the absence of ATP to determine whether competition between nucleotides was occurring at position 75 in standard conditions. Addition of CTP to tRNA-NC was efficient after a typical reaction time of two minutes, giving

tRNA-NCC (Fig. 3-15). CTP addition at position 76 was first seen after two, four and ten minutes for the lysine, methionine, and alanine variants, respectively (Fig. 3-15a, b, c). After ten minutes the lysine variant extended to the third position whereas completion of C76 addition occurred after two hours for alanine and methionine giving all tRNA with 3' endings of -C-C-C. This demonstrated that under standard assay conditions ATP does indeed compete with CTP for addition at position 75.



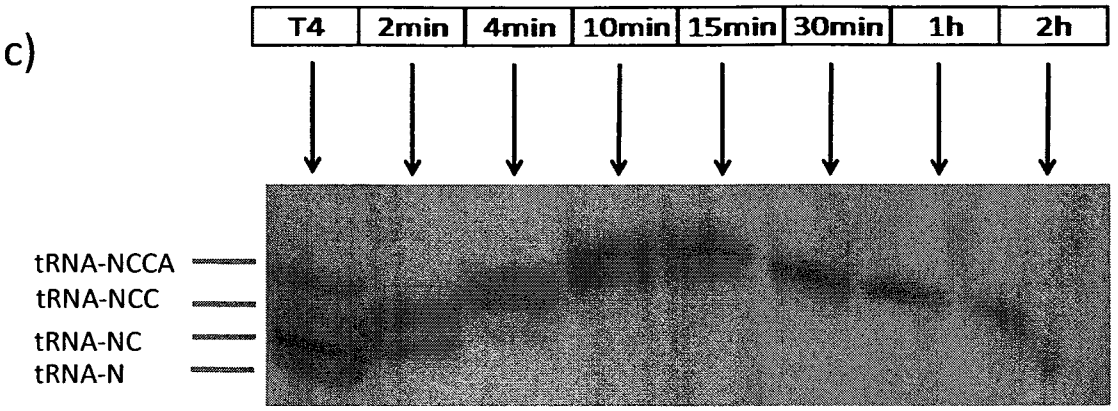


Fig. 3-15. Time course with tRNA-NC in the absence of ATP. a) alanine variant b) lysine variant c) methionine variant. Reactions are performed in standard conditions, in the absence of ATP. Samples of the reaction were removed and terminated at the times indicated. A mixture of all four transcripts are marked as T4 and included as a control. Terminated reactions are analyzed by 12% denaturing gel.

a)

	Length of Reaction (minutes)						
	2	4	10	15	30	60	120
NCCA	-	-	27.3	50.6	89.3	95.8	100
NCC	100	100	72.7	49.4	10.7	4.2	-
NC	-	-	-	-	-	-	-

b)

	Length of Reaction (minutes)						
	2	4	10	15	30	60	120
NCCA	23.7	72.6	100	100	100	100	100
NCC	76.3	27.4	-	-	-	-	-
NC	-	-	-	-	-	-	-

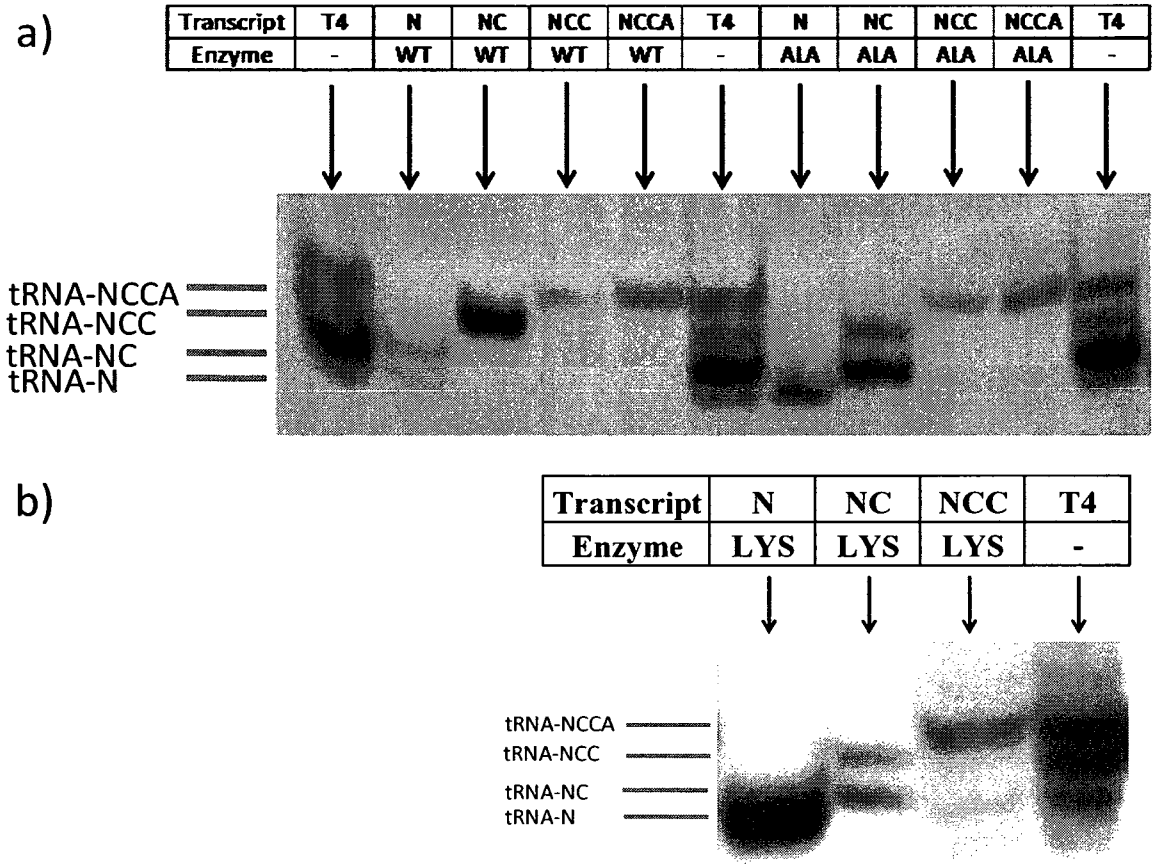
c)

	Length of Reaction (minutes)						
	2	4	10	15	30	60	120
NCCA	-	26.8	60.7	84.8	100	100	100
NCC	16.6	67.7	39.3	15.2	-	-	-
NC	83.4	5.5	-	-	-	-	-

Table 3-8. Percentage of resulting tRNAs from a time course with tRNA-NC in the absence of ATP. a) alanine variant, b) lysine variant, c) methionine variant. Percentage of each product formed after 2, 4, 10, 15, 30, 60, and 120 minutes with tRNA-NC as the substrate. Percentage of each band in each lane is calculated by densitometry using GeneTools (Syngene).

3.4.2.8 Enzyme assays with run-off transcripts in the absence of CTP.

To further address the specificities of all of the enzymes for ATP, each transcript was incubated in the presence of ATP alone. The native enzyme was able to add a single AMP to each position, however, addition to tRNA-N is incomplete after two minutes (Fig. 3-16a), although densitometry reveals that very few do not have ATP added with approximately 98% of the tRNA showing extension. Not surprisingly, the variants did not add A74 to tRNA-N and added inefficiently to tRNA-NC (Fig. 3-16a, b, c). Of more interest, and supporting what had been seen previously with the crude tRNA preparations (Fig. 3-8), both the Ala and Met variants efficiently added A76 to tRNA-NCC (Fig.3-16a, b). Additionally, neither the native enzyme nor the variants can add multiple ATPs,



c)

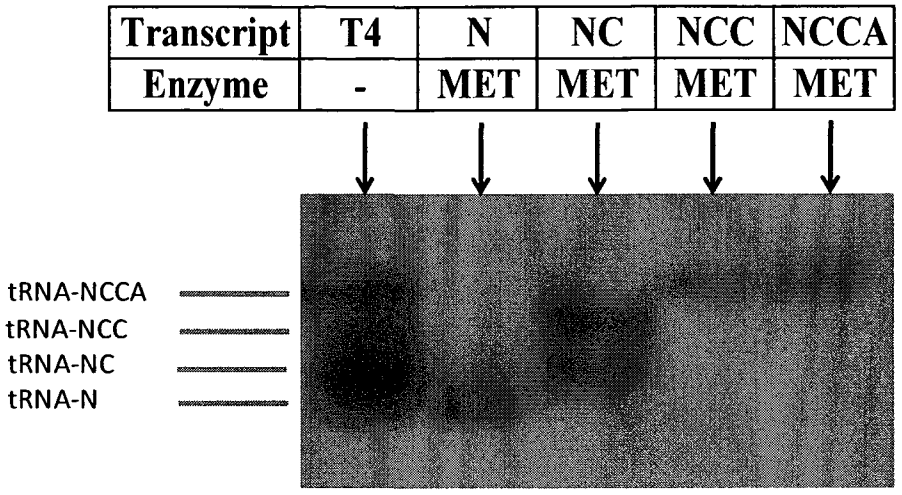


Fig. 3.16. Assays containing transcribed tRNAs in the absence of CTP. a) native enzyme and alanine variant, b) lysine variant, c) methionine variant. Reactions are performed in standard conditions in the absence of CTP. A mixture of all four transcripts are marked as T4 and included as a control. Terminated reactions are analyzed by 12% denaturing gel.

eliminating any possibility that the mutants have become A-adding enzymes or that the second band seen throughout the time course in standard assay conditions (Fig. 3-13) is tRNA-N-C-A-A.

a)

	Substrate							
	Native				Alanine			
	N	NC	NCC	NCCA	N	NC	NCC	NCCA
NCCA	-	-	100	100	-	-	100	100
NCC	-	100	-	-	-	73.5	-	-
NC	97.8	-	-	-	-	26.5	-	-
N	2.2	-	-	-	100	-	-	-

b)

	Substrate		
	N	NC	NCC
NCCA	-	-	96.8
NCC	-	49.9	3.2
NC	-	50.1	-
N	100	-	-

c)

	Substrate			
	N	NC	NCC	NCCA
NCCA	-	-	100	100
NCC	-	55.4	-	-
NC	-	44.6	-	-
N	100	-	-	-

Table 3-9. Percentage of resulting tRNAs obtained from transcribed tRNAs in the absence of CTP. a) native enzyme and alanine variant, b) lysine variant, c) methionine variant. Percentage of each product formed is calculated by densitometry using GeneTools (Syngene).

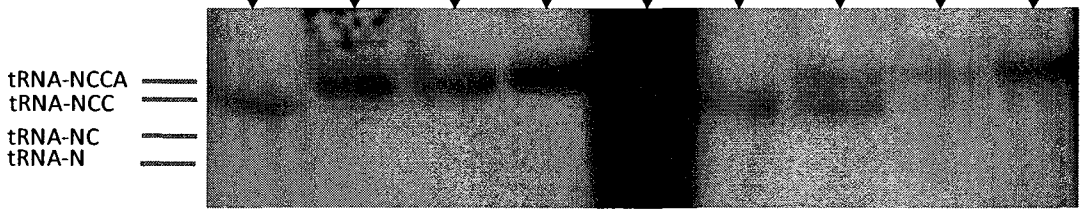
3.4.2.9 Competition assay between ATP and CTP using non-radiolabelled run-off transcribed tRNA-NC and [α-^{32}P] ATP.

To absolutely confirm that nucleotide competition occurs at position 75, non-radiolabelled tRNA-NC was incubated with standard ATP concentrations with increasing concentrations of CTP. To visualize the competition between both nucleotides, radiolabelled ATP was employed. Concentrations of CTP used were 0, 0.1, 0.4, and 1 mM. At 0 mM CTP, ATP is efficiently added to position 75 with all variants (Fig. 3-17). Increasing concentrations of CTP to 0.1 mM, results in the native enzyme extending all tRNA to position 76, where this is only the case for 30% of tRNA for the alanine variant (Fig. 3-17a). The lysine variant adds almost as well as the native with 90% of tRNA extended to position 76 (Fig. 3-17b). Methionine cannot extend past position 75 with CTP concentration levels at 0.1 mM (Fig. 3-17c). In contrast, 0.4 mM CTP allows alanine and lysine to completely extend the substrate tRNA, whereas 50% of tRNA are extended by methionine. Only at 1 mM CTP can methionine extend all tRNAs to position 76 (Fig. 3-17c). These results indicate that competition between both nucleotide substrates does occur and that the two signals seen in the time course assay in standard conditions (Fig. 3-13) represent tRNA-NCA and tRNA-NCCA. With increasing amounts

of CTP, a decrease in tRNA-NCA and an increase in tRNA-NCCA can be seen. Competition between nucleotides occurs in increasing magnitude for lysine, alanine, and methionine, respectively.

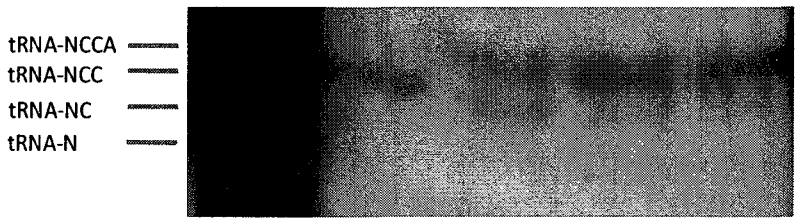
a)

[CTP] (mM)	0	0.1	0.4	1	-	0	0.1	0.4	1
Enzyme	WT	WT	WT	WT	T4	ALA	ALA	ALA	ALA



b)

[CTP] (mM)	-	0	0.1	0.4	1
Enzyme	T4	LYS	LYS	LYS	LYS



c)

[CTP] (mM)	-	0	0.1	0.4	1
Enzyme	T4	MET	MET	MET	MET

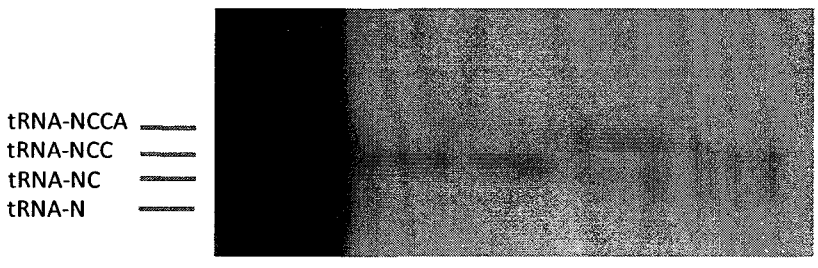


Fig. 3-17. Assays containing non-radiolabelled tRNA-NC and [α-^{32}P] ATP with increasing CTP concentrations. a) Native enzyme and ala variant, b) lysine variant, c) methionine variant. ATP concentrations were kept constant at 1 mM. CTP concentrations were varied and are shown in the table above each lane giving, 0, 0.1, 0.4, and 1 mM. A mixture of all four transcripts are marked as T4 and included as a control. Terminated reactions are analyzed by 12% denaturing gel.

a)

	Native				Alanine			
[CTP]	0 mM	0.1 mM	0.4 mM	1 mM	0 mM	0.1 mM	0.4 mM	1 mM
NCCA	-	100	100	100	-	32.9	97.9	100
NCC	100	-	-	-	100	67.1	2.1	-
NC	-	-	-	-	-	-	-	-

b)

	Lysine			
[CTP]	0 mM	0.1 mM	0.4 mM	1 mM
NCCA	-	88.3	99.4	100
NCC	100	11.7	0.6	-
NC	-	-	-	-

c)

	Methionine			
[CTP]	0 mM	0.1 mM	0.4 mM	1 mM
NCCA	-	-	53.3	95.3
NCC	100	100	46.7	4.7
NC	-	-	-	-

Table 3-10. Percentage of resulting tRNAs obtained from non-radiolabelled tRNA-NC and [α-^{32}P] ATP with increasing concentrations of CTP. a) native enzyme and alanine variant, b) lysine variant, c) methionine variant. Percentage of each product formed is calculated by densitometry using GeneTools (Syngene).

3.4.2.10 A two-step nucleotide addition assay with tRNA-NC

Since C76 addition took two hours for the alanine and methionine variants in the time course assay in the absence of ATP, giving CTP first to the variants using tRNA-NC followed by ATP should allow extension to CCA. Therefore an assay was performed with tRNA-NC in standard conditions where one nucleotide was added to the reaction for the first two minutes while the missing nucleotide was added to the reaction at the two

minute mark. When CTP was the first nucleotide present in the reaction and ATP was added second, the native and lysine enzymes had the majority (85%) of the tRNA processed to position 76 (Fig. 3-18, Table 3-11). Alanine processed 72% of tRNA to a size equivalent to tRNA-NCCA whereas methionine only processed 35% (Fig. 3-18). This would suggest that CTP addition is much slower with the methionine variant compared to the alanine variant. At the two minute mark methionine has not extended all the tRNA-NC to tRNA-NCC and therefore, when ATP is added it can compete better for binding at position 75 as seen in Fig. 3-17c and Table 3-10. The products for this assay are likely to be tRNA-NCCA since after four minutes extension to position 76 has been accomplished with no distinct band for tRNA-NCC. This is in comparison with the ten minutes it takes both the methionine and alanine variants to add CTP to position 76 in the time course assay in the absence of ATP. When ATP is the first nucleotide added, all enzymes demonstrate about the same level of addition (70-80%), except for lysine (50%). The majority of the tRNAs are at an equivalent size to tRNA-NCC. This suggests that all of the variant enzymes can add ATP to position 75 in the absence of CTP as seen in the native enzyme, and that by the time the CTP is added, all the positions are filled with ATP. As seen in the native enzyme, once an ATP is added no further ATPs can be incorporated in the absence of CTP (Fig. 3-16a). It is possible that ATP is too large, *i.e.* purine versus pyrimidine, for another nucleotide to be inserted and explains why extension to position 76 does not occur.

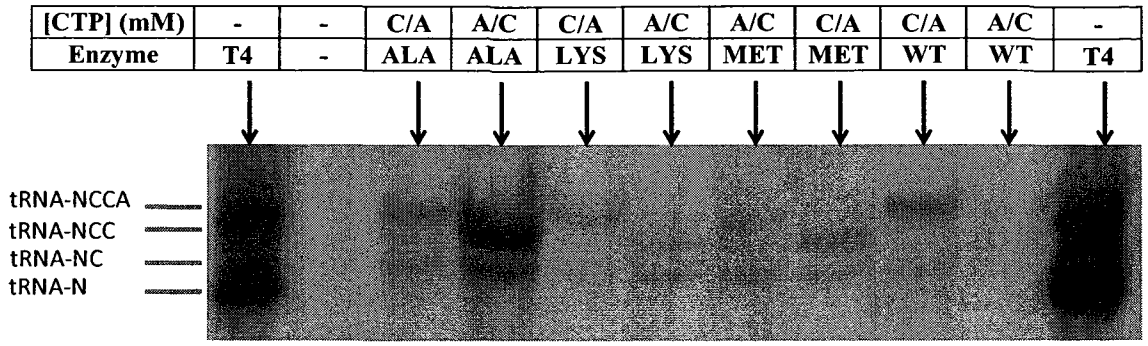


Fig. 3-18. Products resulting from a two-step nucleotide addition assay with tRNA-NC. C/A: CTP first in the reaction, ATP added after two minutes. A/C: ATP first in the reaction, CTP added after two minutes. Assay is performed for two minutes under standard conditions and in the presence of one nucleotide. The other nucleotide is added after two minutes and the reaction is terminated after four minutes.

	Alanine		Lysine		Methionine		Native	
	C/A	A/C	C/A	A/C	C/A	A/C	C/A	A/C
NCCA	72.3	9.4	85.3	-	34.3	-	85.4	-
NCC	-	69	-	44.9	-	69.2	-	78
NC	27.7	21.6	14.7	55.1	65.7	30.8	14.6	22

Table 3-11. Percentage of resulting tRNAs obtained from the two-step nucleotide addition assay with tRNA-NC. C/A: CTP first in the reaction, ATP added after two minutes. A/C: ATP first in the reaction, CTP added after two minutes. Percentage of each product formed is calculated by densitometry using GeneTools (Syngene).

4. Discussion

4.1 Role of arginine 244 in *Candida glabrata* tRNA nucleotidyltransferase

The studies described here were carried out to identify the role that arginine 244 plays in *C. glabrata* tRNA nucleotidyltransferase. Sequence alignments (Fig. 1-3) indicate that this residue is invariant and previous *in vivo* (Fig. 1-8) and *in vitro* experiments (Cho *et al.*, 2007) have indicated its importance. Based on available crystal structures (Li *et al.*, 2002; Augustin *et al.*, 2003; Tomita *et al.*, 2004) and site-directed mutagenesis experiments (Tomita *et al.*, 2004; Cho *et al.*, 2007), at least four possible functions have been suggested for this residue. It may play a role in defining the structure of the protein template for binding CTP or ATP (Cho *et al.*, 2007) and may in fact facilitate the switch in nucleotide recognition from CTP to ATP during nucleotide incorporation (Fig. 1-7)(Li *et al.*, 2002). Changing the corresponding Arg to Glu in the *B. stearothermophilus* enzyme resulted in the loss of the ability to differentiate between CTP and ATP during nucleotide addition (Cho *et al.*, 2007). It may have a potential role in orienting the tRNA in the active site as suggested by crystallographic data of the *A. aeolicus* A-adding enzyme co-crystallized with a tRNA (Tomita *et al.*, 2004). Finally, it has been proposed that this arginine residue has a role in restricting to three the number of nucleotides added at the 3' end of the tRNA (Li *et al.*, 2002; Cho *et al.*, 2007). In fact, it is possible that this arginine residue fulfills any or all of these roles. The experiments described here were designed to provide a better understanding of the possible roles this conserved arginine residue plays in nucleotide addition in tRNA nucleotidyltransferase.

4.2 Arginine 244 is absolutely required for cell viability

The *Candida glabrata* gene *CCA1* was isolated from a genomic library through complementation in *Saccharomyces cerevisiae* (Hanic-Joyce and Joyce, 2002) and cloned into pGEX-2T. Site-directed mutagenesis was used to generate alanine, lysine, and methionine variants. These particular amino acids were chosen based on the following criteria: alanine is the smallest substitution that can be made without destabilizing an α-helix; methionine is somewhat similar in size to arginine but lacks the positive charge; lysine is somewhat similar in size to arginine and retains the positive charge. *In vivo* studies demonstrated that any of these substitutions caused cell death, providing the first direct proof of the vital role of this arginine in enzyme structure or function (Fig. 1-8). Further experiments were necessary to determine whether or not the arginine played a role in structural stability or enzyme activity.

4.3 Changing *C. glabrata* Arg244 to Ala, Lys, or Met does not dramatically alter the structure of the protein

A scheme for the expression and purification of native and variant tRNA nucleotidyltransferases was developed using glutathione-S-transferase affinity chromatography and no significant differences between the native and variant enzymes in terms of overall yields or sensitivity to proteases were observed. The purified proteins were characterized by far-UV circular dichroism (CD) and fluorescence spectroscopy to determine if the basic levels of structure were still intact and to investigate their structural stability.

The CD spectrum of the native enzyme showed typical α-helical signals at 208 nm and 222 nm in good agreement with the 33% and 53% α-helical nature demonstrated

from the available crystal structures of other tRNA nucleotidyltransferases (Li *et al.*, 2002; Augustin *et al.*, 2003; Tomita *et al.*, 2004). Moreover, in these crystal structures the residue corresponding to *C. glabrata* Arg244 was found in an α-helix and we designed our variants to retain the α-helical character of this portion of the protein. Maintenance of this α-helix is supported by the conservation of the overall shape and relative intensity of the CD signal in all three variants (Fig. 3-3). In particular, the alanine and lysine variants showed CD spectra that were virtually indistinguishable from the spectrum of the native protein. The slight variations in signal intensity that were present likely reflected differences in protein concentrations in these samples. Although the methionine variant showed the same general character, it did demonstrate a slightly more intense signal at 222 nm, indicating better helix formation (Creighton, 1989). These differences may reflect the lack of charge in the methionine residue as compared to the lysine and arginine residues. Both lysine and arginine are positively charged amino acids at physiological pH and may slightly destabilize the helix as they are found at the N-terminus of the helix where the portion of the macro dipole is positive (Hecht *et al.*, 1990; Creighton, 1993). Therefore the helix would favour the uncharged methionine at this position. Methionine has been shown to be somewhat favourable for N-capping of an α-helix, whereas lysine and arginine are not (Hecht *et al.*, 1990). Perhaps the alanine variant does not show a similar increase in α-helical signal because the loss of the positive charge alone is not sufficient to show this effect. The large hydrophobic side chain of the methionine variant may also play some role in defining this change in CD spectrum. Overall, changing Arg244 to Ala, Lys, or Met had no dramatic effect on the secondary structure of tRNA nucleotidyltransferase.

Furthermore, as was seen at the level of secondary structure, fluorescence spectroscopy also indicated no major changes in tertiary structure. The native and variant enzymes all showed similar fluorescence spectra (Fig. 3-5). As with the CD spectra, the methionine spectrum was most different from the others and again this may reflect replacing the positively charged arginine residue with a large hydrophobic amino acid. The small change in the emission spectrum for the methionine variant would be consistent with a single tryptophan residue shifting in proximity to a tyrosine residue such that energy transfer from the tyrosine to the tryptophan results (Creighton, 1989). If we look at the positions of available tyrosine and tryptophan residues in the model of *C. glabrata* tRNA nucleotidyltransferase, we see that there are no chromophores in the vicinity of position 244. In fact, there are only three tryptophans within the entire protein (Fig. 1-3) and just two of these are located in the amino-terminal portion of the protein. Given this, it is not surprising that the changes made at position 244 did not result in a drastic change in the structure of the protein as measured by fluorescence spectroscopy. Even if the methionine variant does show a greater change in tertiary structure this is not required for the loss of activity as both the Ala and Lys variants also show a loss of activity as indicated by the cell death phenotype in yeast. Therefore, Arg244 does not play a crucial role in maintaining the structural integrity of tRNA nucleotidyltransferase as changing it to Ala, Met, or Lys does not result in any major change in secondary or tertiary structure at least as measured by CD and fluorescence spectroscopy.

To further define the effects of these substitutions on the structure of tRNA nucleotidyltransferase, thermal denaturation of the native and variant enzymes was carried out. When thermal denaturation was measured as a function of the change in CD

signal at 208 nm with respect to temperature, the resulting curve reflected a two-state cooperative unfolding process without any intermediates for the native and variant enzymes (Creighton, 1993)(Fig. 3-5). While the alanine and methionine variants demonstrate a Tm that is 3-4°C higher than the native enzyme, the lysine variant demonstrates an increase in Tm of only 0.8°C (Table 3-1). As mentioned previously, while the lysine and arginine side chains may slightly destabilize the helix by repulsion with the macro dipole of the helix (Hecht *et al.*, 1990) the methionine and alanine side chains are non-charged and lack the possibility of this unfavourable repulsion interaction, therefore offering a higher degree of stability for the helix. If the unfolding of the enzyme occurs by a two-state process, cooperativity plays a role in the manner in which the enzyme denatures. Any partial unfolding of the enzyme can induce the weakening of other interactions and so forth, causing a continuous unfolding of the enzyme (Creighton, 1993). If the positively charged residues create a slight instability in the helix, then when induced by increasing levels of temperature, the chain reaction of weakening interactions may occur at a faster rate. This would have the opposite effect where methionine stabilizes the helix compared to arginine, and therefore requires a higher temperature to provoke the cascade of weakening interactions. While the apparent increase in thermal stability of the variants cannot be excluded from playing some role in the loss of function of tRNA nucleotidyltransferase, the Lys variant is also inactive (as measured by the *in vivo* phenotype) and shows essentially no difference in Tm as compared to the native enzyme.

In summary, biophysical characterization through circular dichroism and fluorescence spectroscopy have demonstrated that there is no major disruption of the

secondary or tertiary structures of the enzyme upon conversion of Arg244 to Ala, Lys, or Met. Additionally, taken together with the protein purification results, these data suggest that the overall stability of the enzyme had not been dramatically compromised.

4.4 Native and variant tRNA nucleotidyltransferase can use a crude mixture of tRNAs extracted from *Saccharomyces cerevisiae* as a substrate

We have previously shown that changing Arg 244 to Ala, Lys, or Met resulted in cell death *in vivo*. To address why this might be the case we used crude tRNA isolated from *S. cerevisiae* as tRNA substrates for the native and variant enzymes. If the arginine residue was absolutely required, substitution at this position should abolish, in part or completely, nucleotide addition and we would expect reduced activity in an *in vitro* enzyme assay. The standard activity assay for yeast tRNA nucleotidyltransferase used in our lab (Shan *et al.*, 2008) measures the incorporation of a radioactive nucleotide into a heterogeneous population of commercial yeast tRNAs. When the assay was carried out using radiolabelled [α-^{32}P] ATP in the presence of CTP and ATP, the variants tested showed between 54.8% (alanine) and 62.4% (methionine) activity as compared to the native enzyme (Lys omitted)(Fig. 3-8). This confirmed that the arginine at position 244 was important for optimal enzyme activity, however, it suggested that the loss of activity was not very large in relative terms when this position was changed to Ala or Met. This result was particularly puzzling since others in the Joyce lab had shown previously that the *S. cerevisiae* enzyme could lose up to 95% of its activity as measured in this *in vitro* assay and still provide sufficient levels of functional enzyme *in vivo* to keep yeast cells alive (Shan *et al.*, 2008). We further explored why an apparent 50% drop in activity in an *in vitro* assay was correlated to cell death *in vivo* while a different mutation gave a 95%

drop in *in vitro* activity but allowed the cells to remain viable. One possibility is that while we were still recording the incorporation of radiolabel, the enzyme had lost the ability to discriminate between C and A incorporation. As we were only measuring incorporation of radiolabeled A we were only able to say that A was incorporated into the substrate and not at which positions this A was being added. Perhaps we were generating unnatural 3'-termini (*e.g.*, N-A, N-AC, N-CA, N-AA, N-AAA, N-ACA, *etc.*). It has been shown that improper formation of 3'-termini results in tRNAs which either cannot be successfully aminoacylated or recognized by the large ribosomal unit (Sprinzl and Cramer, 1979; Lill *et al.*, 1988; Liu and Horowitz, 1994). If this was the case, that would explain the loss of viability *in vivo*. A second possibility is that the tRNAs that we isolated from yeast represent a population of tRNAs with a mixture of possible 3'-termini, *i.e.*, they may end with N-, N-C, N-CC or N-CCA and that the variant enzymes are unable to recognize some of these ends as substrates and extend them to completion. To try to distinguish between these two possibilities the assays were repeated using radiolabelled CTP instead of ATP or excluding one of the two nucleotide substrates from the reaction. When unlabelled CTP was removed from the reaction, the relative incorporation of labelled ATP into the variants increased with respect to the native enzyme (Fig. 3-8). This suggests that in the variants ATP can compete more efficiently for binding with CTP than in the native enzyme, *i.e.*, the variants cannot discriminate between the two nucleotide triphosphates as well as the native enzyme does. However, when radiolabelled CTP was used instead of radiolabelled ATP the relative activity of the variants was reduced to the level of the boiled enzyme control indicating that CTP was not incorporated at all into these variant enzymes (Fig. 3-8). These results were

essentially the same whether or not unlabelled ATP was included in the reaction (Fig. 3-8). So although it appears that the Ala and Met variants have lost some ability to discriminate between ATP and CTP it appears that the main problem with the variants is in the incorporation of CTP. Two possible explanations at the level of the enzyme for why CTP is not incorporated could be either that CTP itself is not bound or oriented correctly by the enzyme or that the tRNA substrates lacking C residues (*i.e.*, N- and N-C) are not bound or oriented correctly by the enzyme. A third possibility is that the yeast tRNA substrates contain some tRNAs with nucleotides already inserted at positions 74 and 75, such that nucleotides cannot be added at these positions. If one compares the difference in counts per minute incorporated from labelled ATP as compared to those incorporated from labelled CTP for the native enzyme, a 2-fold difference is seen for the incorporation of ATP as compared to CTP (see appendix A). This suggests that while ~75% of the tRNAs are missing the terminal A, less than half of these are also missing at least one C. This is in good agreement with what has been suggested by the supplier (Roche) for individual tRNA species within the mixture of crude tRNA. When four different tRNAs were tested for specific aminoacylation, between 35% and 70% were aminoacylated (depending on the aminoacyl tRNA synthetase used). This reflects the amount of tRNA with an intact CCA terminus suitable for amino acid attachment and indicates that, depending on the specific tRNA, between 30% and 65% do not have a complete CCA sequence. A different experimental approach was required to eliminate the differences in tRNA substrates as a variable and to more precisely explore A and C addition.

4.5 Ability of native and variant enzymes to use specific tRNA substrates

To address the possible reasons for the decrease in activity in the variant enzymes and to try to address why the cells were inviable, homogeneous tRNA substrates ending with -N, -NC, -NCC, or -NCCA were used in *in vitro* enzyme assays. In this case the tRNA substrates were prepared using radioactive nucleotides such that either ATP or CTP addition could be monitored. Furthermore, the addition of one, two, or three nucleotides could be measured as well.

4.5.1 Substrate lacking a 3'-CCA terminus

When a tRNA substrate lacking the entire CCA sequence was used it served as a good substrate for CCA addition with the native enzyme (Fig. 3-11 and Table 3-4). When supplied with tRNA-N, ATP, and CTP the native enzyme extended the tRNA substrate to a size corresponding to the template containing a completed CCA sequence within two minutes (Fig. 3-11, lane 2). In contrast, none of the variant enzymes were able to extend the same template (Fig. 3-11, lanes 5 and 8; Fig. 3-12c, lane 1, Table 3-5c). This immediately suggested why yeast cells carrying these variants are inviable. When the primary transcripts are processed prior to CCA addition they generate a tRNA that is either not recognized or not acted upon by the tRNA nucleotidyltransferase variants. Regardless of whether tRNA substrate recognition or catalysis is reduced, tRNAs containing functional 3'-termini are not generated and protein synthesis cannot occur resulting in cell death.

When the enzymes were presented with only one of the two nucleotides, the native enzyme generated -NCCC and -NA for assays with CTP and ATP, respectively (Fig. 3-11, lanes 3 and 4). The variants, again, did not extend when presented with tRNA

that ended with the discriminator base (Fig. 3-11, lanes 6, 7, 9, and 10). The resulting products from the assays of the native enzyme when one of the two nucleotides was removed are atypical since the native enzyme specifically adds CTPs at positions 74 and 75 and ATP at position 76. However, previous reports using other tRNA nucleotidyltransferases have demonstrated that manipulation of nucleotide concentrations *in vitro* can cause deviations from the normal 3' sequence (*e.g.*, baker's yeast: Rether *et al.*, 1974; *E. coli*: Best and Novelli, 1971; Carre and Chapreville, 1974; Hou, 2000; Seth *et al.*, 2002; rabbit liver: Deutscher, 1972a; Deutscher, 1972b; Deutscher, 1973a; Deutscher, 1973b; *S. shibatae* and *M. jannaschii*: Seth *et al.*, 2002). Similar experiments as performed here in standard assay conditions in the absence of ATP with the baker's yeast, *E. coli*, *S. shibatae*, and *M. jannaschii* enzymes, demonstrated the addition of a -CCC sequence (Rether *et al.*, 1974; Hou, 2000; Seth *et al.*, 2002) as seen here. As well, the absence of CTP in standard assay conditions allowed the baker's yeast and rabbit liver enzymes to add a single ATP to the discriminator base (Rether *et al.*, 1974; Deutscher, 1972a).

From the results utilizing tRNA ending at the discriminator base, arginine 244 has been shown to be involved in nucleotide addition at position 74 and this explains the cell death phenotype *in vivo*. Although these data and those of the experiments involving acid precipitable counts suggest a major role for Arg244 in substrate recognition (either tRNA or CTP) or in the early steps in catalysis, we still cannot exclude an additional contribution of this residue to the switch in nucleotide recognition from CTP to ATP during nucleotide incorporation or in restricting the number of nucleotides added at the 3' end of the tRNA to three.

4.5.2 Substrate lacking a 3'-CA terminus

When under standard assay conditions a substrate containing the first C (position 74) of the CCA sequence was used, the native enzyme had no trouble in completing nucleotide addition to generate a product the same size as the tRNA containing a complete CCA sequence (Fig. 3-12a, compare lanes 3 and 6, Table 3-5). In contrast, all of the variants were less efficient in using this substrate (Fig. 3-12b, lane 3; c, lane 2; d, lane 3). In a standard two minute assay all of the variants showed a mixture of products with less than 40% extended to position 75 and less than 35% extended to position 76 (Table 3-5). It was unknown whether the accumulation of product at position 75 resulted from inefficient ATP (or CTP) addition at position 76, the inability to incorporate CTP but to add ATP inefficiently at positions 75 and 76, or the inability to distinguish between CTP and ATP such that in some cases ATP was incorporated at position 75 with termination of extension at this position.

To distinguish between these possibilities, a time course assay was performed under standard conditions to see if a longer reaction time allowed the variants to complete extension. The variants demonstrated that in the presence of both nucleotides, two lengths of tRNA, equivalent to tRNA-NCC and tRNA-NCCA, can be seen even after a reaction time that spanned two hours (Fig. 3-13a, b, c). In the case of the alanine and methionine variants, extension of the tRNA to position 76 occurred for ~70% and ~35% of the tRNAs, respectively (Table 3-6). In this assay the lysine variant demonstrated high efficiency and extended 95-98% of the tRNA to position 76 with obvious extension even after only two minutes (Fig. 3-13b, Table 3-6). Given what had previously been seen with

the Lys variant in the standard assay (compare Fig. 3-12c, lane 2 to Fig. 3-13b, lane 1) the experiment with the lysine variant will have to be repeated.

The ability of the enzymes with Arg or Lys at position 244 to extend the 3' end of the tRNA to completion (position 76) may come either from their ability to orient the nucleotide for efficient catalysis or through stabilizing the 3' end of the tRNA by hydrogen bonding. The charged amino acids may also be able to discriminate better between nucleotides than can alanine and methionine based on possible hydrogen bonding interactions with the incoming nucleotides. This would be in line with a role in facilitating the switch from CTP to ATP recognition (Li *et al.*, 2002; Cho *et al.*, 2007). If this amino acid was involved in stabilizing the 3'-end of the tRNA prior to CTP addition, then the loss of the interaction by replacing this positively charged residue with an alanine or methionine would decrease catalytic efficiency. Why the alanine variant would allow more efficient extension than the methionine variant may be due to methionine's proximity to the tRNA primer, possibly clashing with the 3' end of the tRNA substrate. Regardless of what is happening at positions 75 and 76 it is important to remember that none of the variants are able to add a nucleotide at position 74, so whatever the arginine residue is doing, it effects addition at position 74 differently from addition at positions 75 and 76.

As the standard reaction could not be chased to completion even within two hours, this suggested that factors other than the inefficient incorporation of CTP may be at play. When the standard assays were repeated in the absence of ATP, CTP was added to tRNA-NC, though inefficiently (4% for lysine and 40-50% for alanine and methionine) as extension was not completed after a typical reaction time of two minutes (Fig. 3-14a,

lane 7; b, lane 3; c, lane 4, Table 3-7). When a standard reaction was carried out with ATP as the sole nucleotide provided, similar inefficient extension (75% for alanine and 50% for lysine and methionine) by one additional position was observed (Fig. 3-16a, lane 8; b, lane 2; c, lane 3, Table 3-9). Taken together these results suggest that replacement of Arg244 by Ala, Met, or Lys results in a reduced ability to catalyze the addition of CTP (and ATP) at position 75 as compared to the native enzyme. This suggests once again that Arg244 may be involved in orienting the incoming nucleotide to facilitate catalysis and that it also stabilizes the 3' end of the tRNA for C75 addition to occur.

When the time course assay was repeated in the absence of ATP, complete extension to position 76 was observed after two hours for the alanine and methionine variants, and after ten minutes for the lysine variant (Fig. 3-15a, b, c). Based on the amount of time it takes for the charged lysine variant and arginine native enzyme to catalyze the addition of CTP compared to the rate with the uncharged Ala and Met substitutions, Arg244 must function to facilitate CTP incorporation. How this occurs remains as speculation, but assisting in the proper orientation of CTP within the active site for proper catalysis to occur may explain this result. A second observation is that in the presence of both nucleotides, the variants cannot complete addition after two hours, but can if ATP is absent from the reaction. This suggests that ATP slows down extension of the 3' end and may compete for binding at position 75 and possibly 76 (Fig. 3-13 compared to Fig. 3-15).

To confirm that competition by ATP does occur at position 75, the ATP concentration was held constant at 1 mM while the CTP concentration was varied. An unlabelled tRNA-NC substrate and [α-^{32}P] ATP were used to indicate extended products.

With increasing CTP concentrations, the band equivalent in length to tRNA-NCC diminished and that which was equivalent to tRNA-CCA increased dramatically (Fig. 3-17a, b, c; Table 3-10). This demonstrated that CTP and ATP compete for incorporation at position 75, and as with the native enzyme, misincorporation of an AMP at this position blocks further extension.

A final study was performed where one nucleotide was introduced into a reaction mixture containing the tRNA-NC template, and after a typical reaction time of two minutes, the second nucleotide was added for an additional two minutes. If CTP was provided first and then ATP was added, 85% of the tRNA was extended to a size equivalent to tRNA-CCA by the native and lysine enzymes. While alanine showed a similar extension efficiency (72%) to the other enzymes, methionine only extended 35% of the substrate to position 76 (Fig. 3-18, Table 3-11). This suggests that CTP addition is slower in the variants which lack the positive charge at amino acid 244 and that addition is much slower with a larger hydrophobic residue (Met) at this position. Perhaps once ATP is added, it competes more efficiently with CTP for binding and addition at position 75 in the alanine and methionine variants than in the enzymes with a positive charge at this position. Given the model proposed based on the crystal structure of the *B. stearothermophilus* enzyme (Cho *et al.*, 2007) this may make sense, as the amino acid corresponding to Arg 244 is thought to coordinate a negatively charged Asp residue (Asp201 in *C.* glabrata) that binds to both ATP and CTP at positions N6 and N4 of their respective base (Li *et al.*, 2002). Perhaps the lysine residue can interact with the aspartic acid residue and allow it to maintain its required position in space for this addition to occur (Fig. 1-7). In contrast, while the alanine residue does not maintain the electrostatic

interaction it does not force the aspartic acid out of position while the larger size of the methionine may cause the orientation of the aspartic acid residue to be altered such that the nucleotides are not bound properly.

In contrast, when ATP was provided first and then CTP was added, the native, alanine 244, and methionine 244 enzymes demonstrated about 70-80% addition up to position 75, whereas lysine 244 showed only about 50% extension to this position (Table 3-11). The majority of the tRNA present after the reaction were equivalent in size to tRNA-NCC (Fig. 3-18). Based on previous results with the native and variant enzymes (Fig. 3-16), ATP can be added only once at position 75 in the absence of CTP. It makes sense therefore, that after two minutes with ATP, all the sites at position 75 are filled with ATP and consequently, the CTP that is added after two minutes cannot be added. This is likely due to the improper orientation of ATP at position 75, which after refolding of the tRNA, the 3'OH is not oriented properly for attack on CTP. Another reason is that after refolding, ATP takes up too much space and does not allow CTP to bind to the active site. This demonstrates that changing arginine 244 has a larger affect on CTP addition than on ATP addition.

The overall conclusions from these data are that arginine plays a role in orienting CTP for addition at position 75, and that it is required to discriminate against ATP binding at this position. This does not eliminate the possibility that tRNA may be stabilized by Arg244 to facilitate faster CTP addition to the 3' terminus of the tRNA. If Arg244 is indeed involved in the stabilization of the tRNA then it appears that tRNA-NC is more readily recognized by the enzyme than is tRNA-N (compare Fig. 3-11 and Fig. 3-12). This suggests that Arg244 can form a greater amount of stabilizing contacts as the

unpaired 3'-portion gets longer, allowing better interactions of the enzyme with the tRNA as the primer grows.

4.5.3 Substrate lacking a 3'–A terminus

When the tRNA substrate lacked only its terminal A residue, it was efficiently extended by all forms of the enzyme under standard assay conditions (Fig. 3-12a, lane 4; b, lane 4; c, lane 3; d, lane 4) or in the absence of CTP (Fig. 3-16a, lanes 4 and 9; b, lane 3; c, lane 4). Interestingly, the alanine and methionine variants seemed to discriminate better against CTP versus ATP than the native enzyme and the lysine variant at this position. For example, with CTP as the only nucleotide, the alanine and methionine variants do not incorporate CTP into position 76 (Fig. 3-14a, c) while the native and lysine variants show some level of extension (Fig. 3-14a, b) within the two minute reaction time. The lysine seems to be as efficient as the native enzyme in adding CTP to position 76 in the absence of ATP to give -CCC, suggesting that a positively charged residue is required to facilitate CTP addition.

4.6 Summary of experimental observations and how they relate to the function of Arg244.

In the present study, the role of arginine 244 in *C. glabrata* tRNA nucleotidyltransferase structure and function was analyzed by amino acid substitution with alanine, lysine, or methionine. Taken together all of these data suggest that Arg244 is important in the functioning of tRNA nucleotidyltransferase and may play multiple roles. One of the major observations was that the variant enzymes could not extend a tRNA from its discriminator base (position 73). This immediately provided an explanation for the *in vivo* phenotype observed and suggested that arginine 244 plays a

direct role in nucleotide addition at position 74 by either directly interacting with the nucleotide or by stabilizing the 3' end of the tRNA within the active site. Both of these possibilities are supported by the available crystal structures. For example, in class II enzymes for which crystal structures are known, the corresponding Arg residue is thought to coordinate with a specific Asp residue important in orienting both nucleotide substrates for catalysis at the active site (see Fig. 1-7 and Cho *et al.*, 2007). Also, a role in coordinating the position of the tRNA at the active site is suggested by the crystal structure of an A-adding enzyme with a tRNA bound (Tomita *et al.*, 2004; Cho *et al.*, 2007). Although this structure shows how the tRNA may interact with that enzyme, it is important to note that this is an A-adding enzyme and the tRNA substrate bound at the active site already has been extended to position 75 (Tomita *et al.*, 2004), so a direct comparison of what is happening with a tRNA ending at position 73 may not be entirely feasible. A second important observation was that incorporation of CTP or ATP seemed to be more efficient as the size of the tRNA template increased. For example, all variants could add C at position 75 (Fig. 3-14 and Fig. 3-15) and A at position 76 (Fig. 3-16) although addition at position 75 seemed to be less efficient than at position 76 (Fig. 3-16). One possible explanation for this observation is that as the tRNA gets extended, it is recognized more efficiently by the enzyme such that subsequent extension steps are easier. This may suggest that the growing 3'-end of the tRNA extends further into the active site such that the tRNA is bound more efficiently by the enzyme or that the extended 3'-end is more accessible for continued nucleotide addition even in the absence of the required arginine at position 244. Again the structure of the A-adding enzyme co-crystallized with tRNA suggests interactions between the tRNA and the enzyme at

multiple positions (*e.g.*, the O2' atom of the ribose of G73 is recognized by an Asn and the O2 and N4 atoms of C75 are recognized by arginine and asparagine residues, respectively (Tomita *et al.*, 2004)) and these interactions may be possible even in the absence of the residue corresponding to Arg244 in the *C. glabrata* enzyme. One further interesting observation was that the Ala and Met variants had a greater efficiency in reducing misincorporation of CTP instead of ATP at position 76 than did the native or Lys variant (compare Fig. 3-14 and Fig. 3-16). This argues against the role of Arg244 being limited to tRNA binding or orientation and supports a role of Arg244 in binding or orienting the nucleotide substrate at the active site. The fact that a positive charge at this position affects nucleotide addition throughout the entire process of making a CCA sequence, signifies that this residue either interacts directly with the base and the residue and/or the tRNA or coordinates an amino acid that is involved in one or more of these interactions. Since the lysine variant cannot add nucleotides to position 74 (Fig. 3-12c, lane 1; Fig. 3-14b, lane 2; Fig. 3-16b, lane 1), this suggests that having a positively charged side chain is not sufficient for CTP addition as only with the specific side chain of arginine does nucleotide addition occur at this position. Analysis through mutagenesis of the amino acids which are said to form the nucleotide recognition apparatus of class II enzymes (Glu153, Asp154, Arg157, in *B. stearothremophilus* see Fig. 1-5), demonstrates that C75 and A76 addition are abolished when Asp154 and Arg157 are changed to asparagine and glutamine respectively, although surprisingly, C74 addition can still take place (Cho *et al.*, 2007). This suggests that the protein recognition and binding apparatus (as determined by Li *et al.*, 2002, see Fig. 1-5) is only required for positions 75 and 76 of the 3' CCA sequence. The data presented here suggests the possibility that the first CTP

is bound in a different way perhaps involving interactions between the guanidinium group of arginine 244 and the O2 and N3 positions of the CTP base.

An explanation why the nucleotide binding apparatus (made up of the (E/D)DxxR motif (Fig. 1-5)) is not required for C74 addition may stem from the distance of the bound CTP to the tRNA primer. If CTP is always bound to the same location of the active site, then the tRNA primer has a greater distance to overcome for the first CTP addition as compared to the second CTP addition, since the tRNA substrate in the latter case will have acquired an additional base, extending the length of the primer. If the distance was too great between the first bound CTP and the tRNA, a variation of the CTP binding process may involve arginine 244 in conjunction with the phosphate backbone of the tRNA and a β-turn facing the arginine residue in the active site, as seen with a vital arginine residue in class I CCA-adding enzymes (Tomita *et al.*, 2006; Toh *et al.*, 2008). The presence of arginine 244 would then likely help increase the rate of catalysis for C74 addition since this would shorten the gap between the primer and the first incoming base, allowing the phosphates to be bound and properly oriented by the divalent metal ions.

4.7 Potential conservation of mechanism between class I and class II enzymes.

It has been proposed that class I and class II enzymes arose from an ancient common ancestor, but have diverged significantly in sequence over time (Okabe *et al.*, 2003). In fact, it appears that the mechanisms also have diverged such that class I CCA-adding enzymes require the aid of tRNA to specifically bind ATP and CTP, whereas class II enzymes only require specific amino acids in the neck region of the enzyme which rotate to accommodate the incoming nucleotide (Okabe *et al.*, 2003). With this in mind it is interesting to speculate that the amino acid corresponding to Arg244 may

function in a similar manner in both of these classes of enzymes. Although there is no sequence similarity between class I and class II enzymes, if one overlays the active site regions of sample enzymes from these two classes one sees a great degree of conservation in higher order structure (Fig. 4-1). The conserved residues shown to be involved in CCA addition in class I enzymes (including those corresponding to Arg244) are spatially conserved in both classes, further suggesting that the mechanistic approaches of both classes of enzymes may be highly similar (Fig. 4-1). This is particularly intriguing given that the crystallographic data, for the *A. fulgidus* class I enzyme, suggests that a conserved arginine residue (spatially conserved with the *C. glabrata* Arg244) binds and interacts with the incoming nucleotide during catalysis (Tomita *et al.*, 2006). When tRNA-NC74 is bound in the *A. fulgidus* enzyme, the C74 base is pinned between the discriminator base and a specific β-turn (Tomita *el.*, 2006; Toh *et al.*, 2008). The conserved arginine residue changes its conformation with respect to the discriminator base to form bipartite hydrogen bonds with the O2 and N3 positions of the incoming CTP. Through these specific hydrogen bonding interactions the arginine residue is able to select CTP and discriminate against ATP. Moreover, the presence of this large amino acid at this location also restricts access to the active site, preventing the binding and addition of the larger ATP (purine versus pyrimidine) to position 75. This may explain why changing Arg244 to Ala, Met, or Lys would allow ATP to compete for binding to

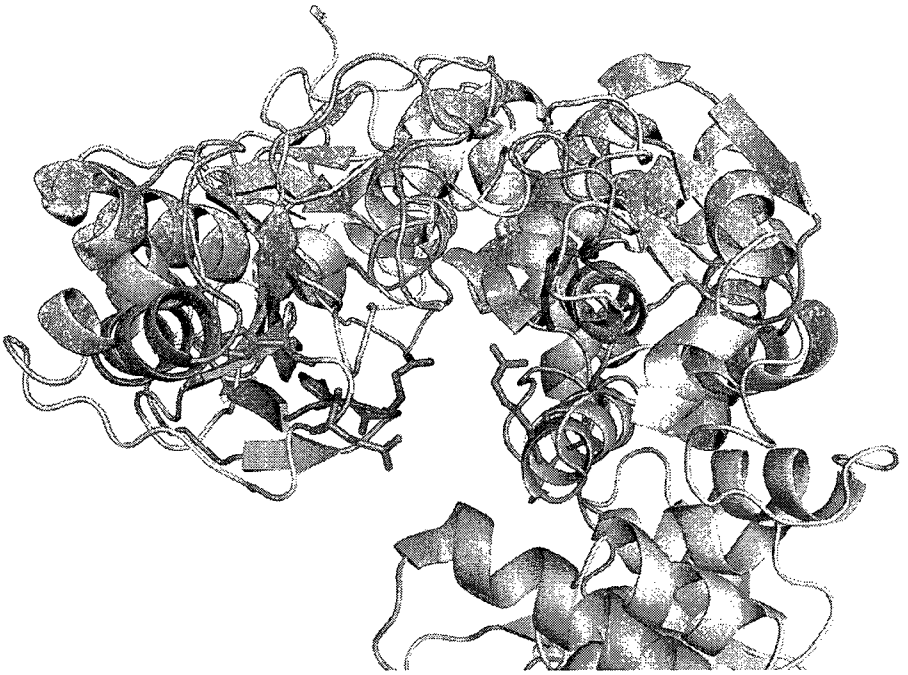


Fig. 4-1. Model of the superposition of the binding and catalytic domains of *H. sapiens* (class II) and *A. fulgidus* (class I) tRNA nucleotidyltransferases. *H. sapiens* is in yellow and *A. fulgidus* is in blue. Residues (Glu96, His97 and Arg224 show in green for *A. fulgidus* and Glu106, His108, and Arg237 shown in red for *H. sapiens*) are conserved and located in the same spatial context in both enzymes and have been found to play a direct role in the addition of CCA in the class I *A. fulgidus* enzyme. The superpositioning of the models representing the crystal structures of *H. sapiens* and *A. fulgidus* tRNA nucleotidyltransferases was done using LSQMAN (Kleywegt, 1996). PyMol was used to visualize the models (Delano, 2009).

position 75 in the *C. glabrata* enzyme (Fig. 3-17). Indeed, changing the conserved arginine residue to alanine in the *A. fulgidus* enzyme reduced C75 incorporation and may be explained by the lack of discrimination against ATP (Toh *et al.*, 2008). Moreover, the available class I crystal structures show that this conserved arginine stabilizes and orients the tRNA in the active site through hydrogen bonding interactions with the discriminator base (Tomita *et al.*, 2006). This is in good agreement with one of the roles proposed for Arg244 here and would suggest that if this arginine was lost, the 3'end of the tRNA would not be oriented properly for C74 addition.

4.8 A proposed mechanism involving Arg244

I propose that arginine 244 in the *C. glabrata* enzyme plays multiple roles in tRNA nucleotidyltransferase depending on the position of the tRNA that requires nucleotide addition. While the available crystal structure of the *B. stearothermophilus* tRNA nucleotidyltransferase suggests that the residue corresponding to *C. glabrata* Arg244 is involved in coordinating an aspartic acid residue which interacts with the incoming CTP or ATP residues (at least prior to addition at positions 75 and 76), I suggest that the first incoming CTP would be recognized directly by Arg244 through Watson-Crick interactions with the O2 and N3 positions of the CTP base such that the distance between the nucleophilic 3'OH of the tRNA primer is in good proximity to the phosphates of the nucleotide and both can be oriented by the bound divalent metal ions. The Watson-Crick interactions would not be of the appropriate pattern for ATP and therefore, it is blocked from addition to position 74. Once addition of the CTP to position 74 occurs, the newly synthesized 3'end would refold to facilitate the binding of the second CTP to the (E/D)DxxR sequence as suggested by the available crystal structures (Li *et al.*, 2002; Tomita *et al.*, 2004). After addition of this first nucleotide the extended tRNA could now form additional interactions with arginine 244 and with a β-turn facing the opposite side of the active site from the arginine 244 (as suggested by Tomita *et al.*, 2006) to stabilize the 3'end of the tRNA. The interactions of both the β-turn and the arginine allow limited access to the active site and therefore discriminate against the incorporation of the larger ATP molecule. The second CTP is then added as suggested by the crystal structure of the *B. stearothermophilus* enzyme where the amino acid corresponding to Arg244 now plays a role in coordinating D201 of the (E/D)DxxR motif.

Once the second CTP is added, the tRNA once again refolds inducing a conformational change of the enzyme. This conformational change allows the first acidic residue and the arginine residue of the (E/D)DxxR sequence to rotate such that ATP is now a suitable substrate (compare the left and right panels in Fig. 1-5).

4.9 Conclusion

Of the roles proposed for arginine 244 (Li *et al.*, 2002; Cho *et al.*, 2007) three are supported by this study: 1) recognizing substrates (Li *et al.*, 2002), 2) facilitating a switch from CTP to ATP recognition (Cho *et al.*, 2007), and 3) orienting the growing 3' end of tRNA in the active site (Cho *et al.*, 2007). No evidence from this study suggests that the arginine residue assists in limiting the extension of the tRNA primer to three nucleotides although in previous studies this has been shown to involve at least three different amino acids (corresponding to R244, V247, and E248 in *C. glabrata*) (Cho *et al.*, 2007). Here I will summarize the role of Arg244 in the complete synthesis of a functional CCA terminus. It first aids in orienting the first incoming CTP through direct Watson-Crick-like interactions between the amino acid side chain and the O2 and N3 positions of cytidine as seen for C75 addition in *A. fulgidus* (Tomita *et al.*, 2006). These specific interactions would allow the arginine residue to discriminate against ATP at position 74 since the purine requires a different hydrogen bonding pattern. Once the first C has been added to the tRNA, the tRNA refolds and the arginine residue switches interactions from the C74 base to those which stabilize the tRNA and Asp201 of the (E/D)DxxR binding sequence. This allows Arg244 to simultaneously assist in the proper orientation of the 3'OH for the next addition and facilitate the binding of the incoming nucleotide. The second CTP can now enter the active site and bind to the (E/D)DxxR

motif. The hydrogen bonding interaction between arginine 244 and the tRNA restricts access to the active site and allows discrimination against the larger nucleotide, ATP. After addition of CTP, the tRNA refolds once again. The refolding of the tRNA induces a conformational change of the enzyme, whereby the Glu/Asp and Arg residues in the (E/D)DxxR motif rotate to accommodate and bind ATP and no longer can form the proper interactions to recognize CTP. Once the ATP is added to the final position, the completed tRNA disassociates from the enzyme.

This proposed mechanism would imply the existence of another binding site besides the (E/D)DxxR motif. This is consistent with C74 addition occurring even in the absence of the (E/D)DxxR motif, but is prevented with the substitution of arginine 244. This mechanism can also explain how ATP is prevented from binding to positions 74 and 75 and how changing arginine 244 would result in a loss for nucleotide specificity. Finally, ATP is the preferred substrate at position 76 since the (E/D)DxxR motif has rotated to specify two hydrogen bonds instead of three and therefore, the binding interaction is more stable with ATP than if CTP were to bind (Fig. 1-5).

As all tRNA nucleotidyltransferases are thought to have arisen from a common ancestor (Okabe *et al.*, 2003), perhaps the functions of this specific arginine residue have been conserved throughout evolution. Aspects of the proposed mechanism mentioned here have been observed in one or the other class of tRNA nucleotidyltransferases and leads one to suggest that the overall processing of tRNA might not differ all that much between classes of CCA-adding enzymes.

4.8 Future Work

Certain experiments would strengthen a few of the suggested roles that arginine may play in *C. glabrata* tRNA nucleotidyltransferase. Obtaining kinetic parameters for binding (K_M) of different tRNA substrates would allow us to determine if indeed Arg244 plays a stabilizing role through interactions with the growing 3' end of tRNA. Performing kinetic studies to obtain binding information for CTP, may reveal whether or not arginine 244 interacts with the first incoming nucleotide before addition instead of the proposed protein template for class II enzymes. Determining the catalytic efficiency (k_{cat}/ K_M) for the addition of CTP would also demonstrate that the conserved arginine is required for addition of this particular nucleotide and reinforce the proposed role of properly orienting CTP for catalysis. Mutational analysis of the protein template residues Asp201 and Arg204 (Asp154 and Arg157 in Fig. 1-5 and 1-7) may confirm that C74 addition can occur without the presence of these residues, whereas C75 and A76 addition requires them. Other possible residues that assist in CCA addition may be located within a β-turn in class II enzymes in the region of 101-115 of *C. glabrata*. Mutational analysis of a serine and a histidine residue found within this loop at position 110 and 112 of the *C. glabrata* enzyme may determine if these residues play similar roles as those found in *A. fulgidus* at positions 96 and 97. This may provide a better understanding of the process by which tRNA is stabilized within the active site, such that the absence of these residues may affect addition at certain or all steps of the CCA adding process, possibly giving additional support for a common mechanism of addition between both classes of tRNA nucleotidyltransferases.

The outcome of these studies would certainly provide more insight on how the enzyme catalyzes -C-C-A addition to the 3' end of tRNA and more specifically, how well the roles proposed here would face up to additional scrutiny of this unique enzymatic process.

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6. Appendix

1) Calculation of the total amount of moles of [α-^{32}P] ATP in a reaction

$$[\alpha\text{-}^{32}\text{P}]\ \text{ATP}\ (\mu\text{Ci}) = \frac{\text{Volume of } [\alpha\text{-}^{32}\text{P}]\ \text{ATP}\ (\mu\text{L})}{\text{Concentration of stock } [\alpha\text{-}^{32}\text{P}]\ \text{ATP}\ (\mu\text{Ci}/\mu\text{L})}$$

$$[\alpha\text{-}^{32}\text{P}]\ \text{ATP (mol)} = \frac{[\alpha\text{-}^{32}\text{P}]\ \text{ATP (Ci)}}{\text{Specific Activity (Ci/mol)}}$$

2) Calculation of total counts per minute for [α-^{32}P] ATP in a reaction

$$[\alpha\text{-}^{32}\text{P}]\ \text{ATP (cpm/mmol)} = \frac{\text{amount of } [\alpha\text{-}^{32}\text{P}]\ \text{ATP before the reaction cpm}}{\text{amount of moles of } [\alpha\text{-}^{32}\text{P}]\ \text{ATP}}$$

3) Calculating the total counts per minute (cpm) obtained from precipitable count assays

Total cpm from assays = cpm / ^{32}P Decay factor

$$\text{4) tRNA with } [\alpha\text{-}^{32}\text{P}]\ \text{ATP added (mol)} = \frac{\text{total cpm from assays (cpm)}}{\text{total } [\alpha\text{-}^{32}\text{P}]\ \text{ATP (cpm/mol)}}$$

5) Amount of total tRNA with ATP added (mol) =

$$\text{tRNA with } [\alpha\text{-}^{32}\text{P}]\ \text{ATP added (mol)} \times \frac{\text{ATP (mol)}}{[\alpha\text{-}^{32}\text{P}]\ \text{ATP (mol)}}$$

6) Concentration of tRNA with ATP added (M) =

$$\frac{\text{Amount of total tRNA with ATP added (mol)}}{\text{Volume of the reaction mix (L)}}$$

Sample Calculation:

1) 0.2 μL [α-^{32}P] ATP x 10 μCi/μL = 2 μCi of [α-^{32}P] ATP

2 x10^{-6} Ci / 3000 Ci/mmol = 6.67 x 10^{-10} mmol = 6.67 x 10^{-13} mol

2) 2 μCi of [α-^{32}P] ATP gives 4399245 cpm

4399245 cpm / 6.67 x10^{-13} mol = 6.6 x10^{18} cpm/mol of total [α-^{32}P] ATP

3) Experiment triplicate #1: 25055 cpm / 0.9527 (1 day factor) = 26299 cpm
triplicate #2: 21862 cpm / 0.9527 (1 day factor) = 22947 cpm
triplicate #3: 13777 cpm / 0.8237 (4 days factor) = 16725 cpm

Average = (26299 + 22947 + 16725)/3 = 21990 cpm

4) 21990 cpm / 6.6 x10^{18} cpm/mol = 3.33 x10^{-15} mol

5) 3.33 x10^{-15} mol x $\frac{1 \times 10^{-7} \text{ mol ATP}}{6.67 \times 10^{-13} \text{ mol [}\alpha\text{-}^{32}\text{P] ATP}}$ = 5 x10^{-10} mol of total tRNA

6) 5 x10^{-10} mol / 1 x10^{-4} L = 5 x10^{-6} M or 5 μM of tRNA with ATP added

7) 5 μM / 20 μM x 100 = 25% of tRNA in the reaction had ATP added

ATP	+	+	-	+
CTP	+	+	+	-
[α-^{32}P] ATP	+	-	-	+
[α-^{32}P] CTP	-	+	+	-
tRNA (x10^10 mol)	5	2.67	2.43	4.11
tRNA (μM)	5	2.67	2.43	4.11
Percent processed	25.0%	13.4%	12.2%	20.6%

Table 6-1. Summary of the amount of label transferred to tRNA in precipitable counts assays for the native enzyme.