A Comparison of Mitochondrial and Cytosolic tRNA Nucleotidyltransferase Enzymes from *Triticum aestivum*

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ABSTRACT

A comparison of mitochondrial and cytosolic tRNA nucleotidyltransferase enzymes from *Triticum aestivum*

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The enzyme ATP (CTP): tRNA-specific tRNA nucleotidyltransferase is required for the synthesis of functional tRNAs in eukaryotic cells. In yeast, a single gene provides this enzyme which is required for the biosynthesis of mature tRNAs in both the nuclear/cytosolic and mitochondrial compartments. To determine if this enzyme is also shared between multiple intracellular compartments in plants, we isolated and characterized the mitochondrial tRNA nucleotidyltransferase from *Triticum aestivum*, along with its cytosolic counterpart. Our purification scheme involved ammonium sulfate fractionation and chromatography on anion exchange, hydroxylapatite and affinity columns. Although the two enzymes share some common features, they also show differences that suggest they are distinct proteins. Specifically, the two enzyme activities elute from hydroxylapatite columns at different ion concentrations and show different molecular masses on gel filtration chromatography. Additionally, the two enzymes showed different levels of activity in the presence of manganese ions.

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Lastly, I would like to dedicate this manuscript to my mom and dad for their endless love and support.

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LIST OF ABBREVIATIONS

A	adenosine
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	cytidine
CCA	cytidine-cytidine-adenosine
cDNA	complementary deoxyribonucleic acid
CMP	cytidine monophosphate
CNBr	cyanogen bromide
CPM	counts per minute
CTP	cytidine triphosphate
DEAE	diethylaminoethyl
dH_20	distilled water
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetracetic acid
HA	hydroxylapatite
kDa	kilo daltons
mRNA	messenger ribonucleic acid
nm	nanometers
P100	pellet resulting from 100 000 X g centrifugation
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonlyfluoride
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S100	supernatant resulting from 100 000 X g centrifugation
SDS	sodium dodecyl sulfate
tRNA	transfer ribonucleic acid
V	volts
X g	times gravitational force

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INTRODUCTION

1. TRANSFER RIBONUCLEIC ACIDS

Transfer RNA (tRNA) molecules serve as interpreters of the genetic code. They are the crucial link between the sequence of nucleotides in mRNA and the sequence of amino acids in a polypeptide They are adaptor molecules in protein synthesis and are the means by which free amino acids are brought to the ribosome and matched up to the correct codons of the mRNA. Transfer RNAs also function as primers for reverse transcriptase (Dahlberg, 1980) and in cell wall biosynthesis (Zachau, 1978). In order for tRNA to fullfill the role of adaptor, every cell must contain at least twenty tRNA species (one for every amino acid), and each of these tRNA molecules must be able to recognize at least one mRNA codon.

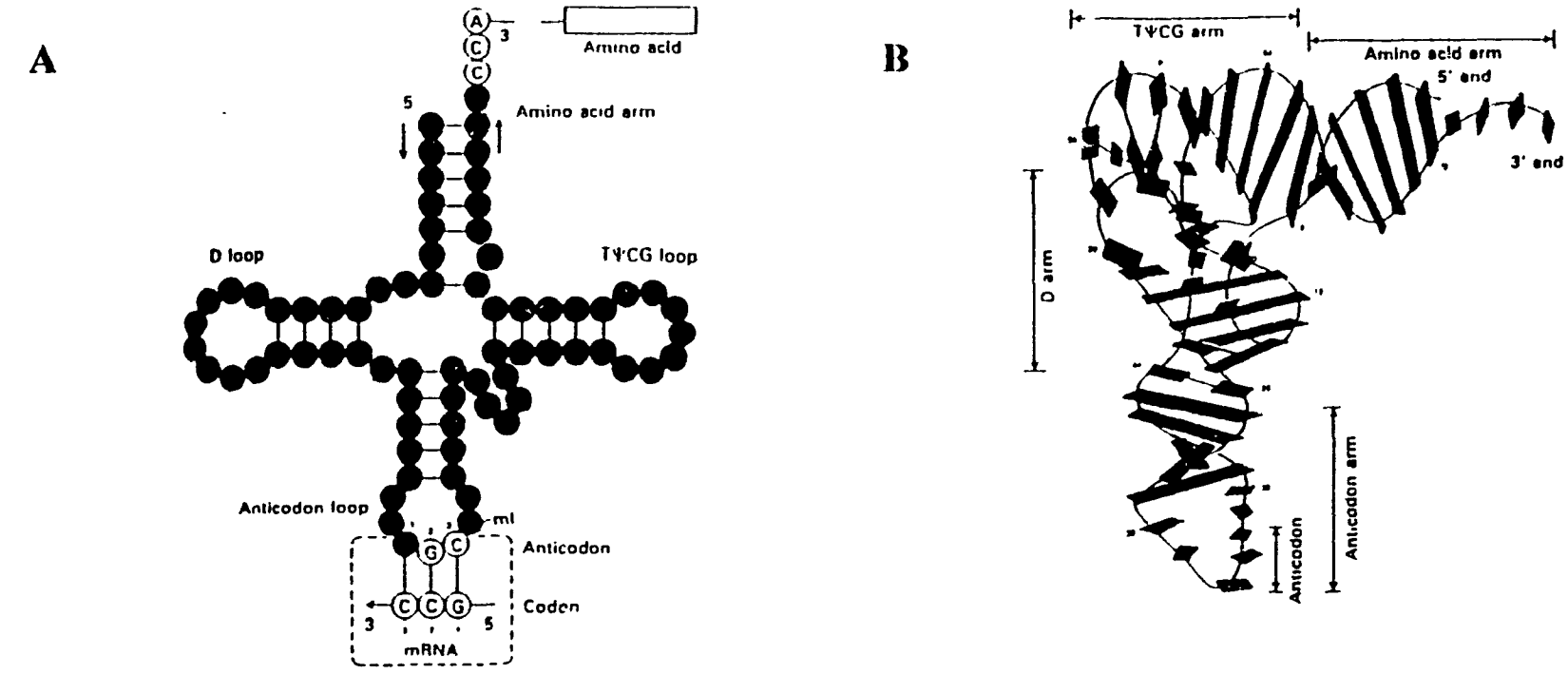
In eukaryotes, protein synthesis occurs in multiple cellular compartments. Since both nuclear and mitochondrial DNA generally contain tRNA genes (Moran and Scrimgeour, 1994), transfer RNAs are found in multiple cellular compartments.

A) All tRNA molecules have a similar structure.

The nucleotide sequences of different tRNA molecules from many organisms have been determined. Despite diversity in their primary structures, a typical tRNA molecule contains between 73 and 95 nucleotides (Moran and Scrimgeour, 1994). The sequences of almost all analyzed tRNA molecules are compatible with the cloverleaf secondary structure first proposed by Holley *et al.* (1965) . This cloverleaf structure is defined by hydrogen bonded stems and single-stranded loops (Figure 1) . A majority of the bases that make up a tRNA molecule are involved in hydrogen-bonding interactions. Hairpin folds bring bases

FIGURE 1

SECONDARY AND TERTIARY STRUCTURE OF TRANSFER RIBONUCLEIC ACIDS



The common cloverleaf secondary structure shared by most transfer ribonucleic acids (A) The common L-shaped tertiary structure shared by most transfer ribonucleic acids (B) (Darnell *et al* , 1990)

of the same chain into a double-helical arrangement where short stretches of nucleotides are complementary to one another However, some bases do not pair internally and are available to interact with other molecules during the functioning of tRNA in protein synthesis (Moran and Scrimgeour, 1994) In addition to the base-pairing interactions in the stems, there is a series of additional hydrogen bonds that fold the cloverleaf into a stable tertiary structure with a roughly L-shaped appearance (Figure 1). This common tertiary structure is required by tRNAs to bind to a common recognition site during protein synthesis (Stryer, 1988). While this cloverleaf model seems to have been conserved through evolution, different tRNAs are characterized by subtle conformational differences linked to variabilities in the tertiary interaction networks and in the sequence arrangement and length of their D-loops and variable regions (Steinberg and Cedergren, 1995). It has been suggested that this conformational variability plays a major role in many specific functions of individual tRNAs (Grosjean *et al.*, 1996).

Although there is much variability among tRNA sequences, certain positions are conserved from one tRNA to another For instance, the 3' end of a tRNA always terminates in a CCA (cytidylic acid, cytidylic acid, adenylic acid) sequence. This CCA terminus serves an important role in protein synthesis as it is the site of amino acid attachment The 5'-end always carries a 5'-terminal monophosphate group and is often guanylic acid (Stryer, 1988). An interesting aspect of all tRNA sequences is their high content of unusual bases (bases other than adenine, guanine, cytosine, or uracil) (Stryer, 1988) Many of these unusual bases differ from "normal" bases by the presence of one or more methyl groups (Stryer, 1988). Although the function of most of the unusual bases is not yet clear, some have been shown to play important regulatory roles in tRNA function (Moran and Scrimgeour, 1994). This is conceivable since methylation imparts a hydrophobic character to some regions of tRNAs, which may be important for their interaction with synthetases and ribosomal proteins (Moran and Scrimgeour, 1994). Also, methylation may prevent the formation of certain base pairs by rendering some of the bases

accessible for other interactions (Björk and Kohli, 1990). Recently, it has been suggested (Steinberg and Cedergren, 1995) that the presence of dimethylguanosine at position 26 in some cytosolic tRNAs may prevent an unusual folding pattern found in some mitochondrial tRNAs.

2. MATURATION OF tRNAs

Mature tRNA molecules are generated in both eukaryotes and prokaryotes by the processing of primary transcripts. Although tRNA maturation in prokaryotes and eukaryotes shares many common features, there are differences in the enzymes and processes involved among different organisms.

A) Generation of a mature 5' terminus

In both eubacteria and eukaryotes, the 5'-leader sequence removal is mediated by RNase P (Altman, 1984). In prokaryotes such as *E.coli*, most tRNA primary transcripts contain more than one tRNA precursor. Approximately half of these primary transcripts also contain precursors of rRNA or mRNA. The monomeric tRNA precursors are cleaved from the large primary transcripts and trimmed to their mature lengths by a variety of ribonucleases (Moran and Scrimgeour, 1994). The 5' end of a tRNA precursor is generated by the activity of the endonuclease RNase P (Moran and Scrimgeour, 1994). Portions of the primary transcript that will become mature tRNA molecules exhibit tertiary structures that are recognized by RNase P. RNase P can recognize tRNA substrates at different stages of processing, but tRNAs with processed 3' termini were shown to be the best substrates (Altman, 1984). This endonuclease catalyzes the cleavage of the primary transcript on the 5' side of each tRNA precursor releasing tRNA precursors with mature 5'

ends. Ribonuclease P serves this function not only in *E.coli* but also in all organisms studied to date (Deutscher, 1984).

B) Generation of a mature 3' terminus

Removal of the 3'-trailer sequence occurs by different means in eukaryotes and eubacteria In eukaryotes the 3'-terminus of tRNA is generated by a single endonucleolytic cleavage, whereas in eubacteria, an endonucleolytic cleavage is made some distance away from the 3'-terminus and is followed by exonucleolytic trimming (Deutscher, 1984). In some cases, the primary tRNA transcript is short, and further processing involves exonucleolytic digestion from the 3' end. In other case, a free 3' end of a tRNA precursor can be generated by RNase P cleavage of an adjacent tRNA or by other endonucleases, such as RNase F in *E.coli*, that release a monomeric tRNA precursor from a larger transcript (Moran and Scrimgeour, 1994). Processing the 3' ends of tRNA precursors in *E.coli* involves several different exonucleases (Deutscher, 1984). RNase D catalyzes the removal of individual nucleotide residues from the 3' end of a monomeric tRNA precursor until it reaches the CCA sequence. Like RNase P activity, RNase D activity can be lost without inhibiting the growth of a cell (Asha *et al.*, 1983), which indicates that the activity can be replaced by the activity of other RNases. RNase BN and RNase T, for example, seem to carry out reactions that also generate mature 3' ends (Deutscher, 1984). Although the signal that halts digestion by RNase D has not been characterized, it has been suggested that the tertiary structure of tRNAs prevents further cleavage after the 3' end has been generated (Deutscher, 1984).

C) Base modifications

In both prokaryotes and eukaryotes, mature tRNA molecules exhibit a greater diversity of covalent modifications than any other class of RNA molecule. The different types of modifications identified to date include ribosylations, isopentenylations and most commonly methylations (Björk *et al.*, 1990). Modified nucleosides are contained in tRNA from archaebacteria, eubacteria and eukaryotes (Bjork, 1995), however, the number and variety of modified nucleosides identified varies between kingdoms. Nucleoside modification is greater in eukaryotes where more than 80 modifications have been identified (Björk and Kohli, 1990) Greater variation in covalent modification of tRNAs occurs in eukaryotes than in prokaryotes, and only a few modifications are common to all organisms (Moran and Scrimgeour, 1994). Like other enzymes involved in processing of tRNA precursors, enzymes involved in the covalent modification of tRNA precursors seem to recognize elements of both primary and tertiary structure near the target nucleotide residue (Björk *et al.*, 1990). In both prokaryotes and eukaryotes, the targets of covalent modifications are generally those regions of the tRNA molecule that do not form intramolecular base pairs. These base modifications, while not essential for cell viability (Björk *et al.*, 1987), seem to play an important role in normal tRNA function These modifications may increase translational efficiency and fidelity as well as increasing the conformational stability of tRNA molecules (Björk *et al*, 1987). Recently it has been proposed (Grosjean *et al.*, 1996) that the function of some modification enzymes may be to promote and stabilize the folding of precursor tRNAs into a correct tertiary structure Furthermore, it was suggested (Grosjean *et al.*, 1996) that the tRNA processing pathway may in fact be a tRNA folding pathway in which certain modification enzymes ensure production of only correctly and completely folded tRNAs to be used during protein synthesis. In fact, Steinberg and Cedergren (1995), recently proposed such a role for N^2,N^2-dimethylguanosine-specific tRNA methyltransferase which catalyzes the modification of a guanosine residue at position 26 in eukaryotic and archaen tRNAs.

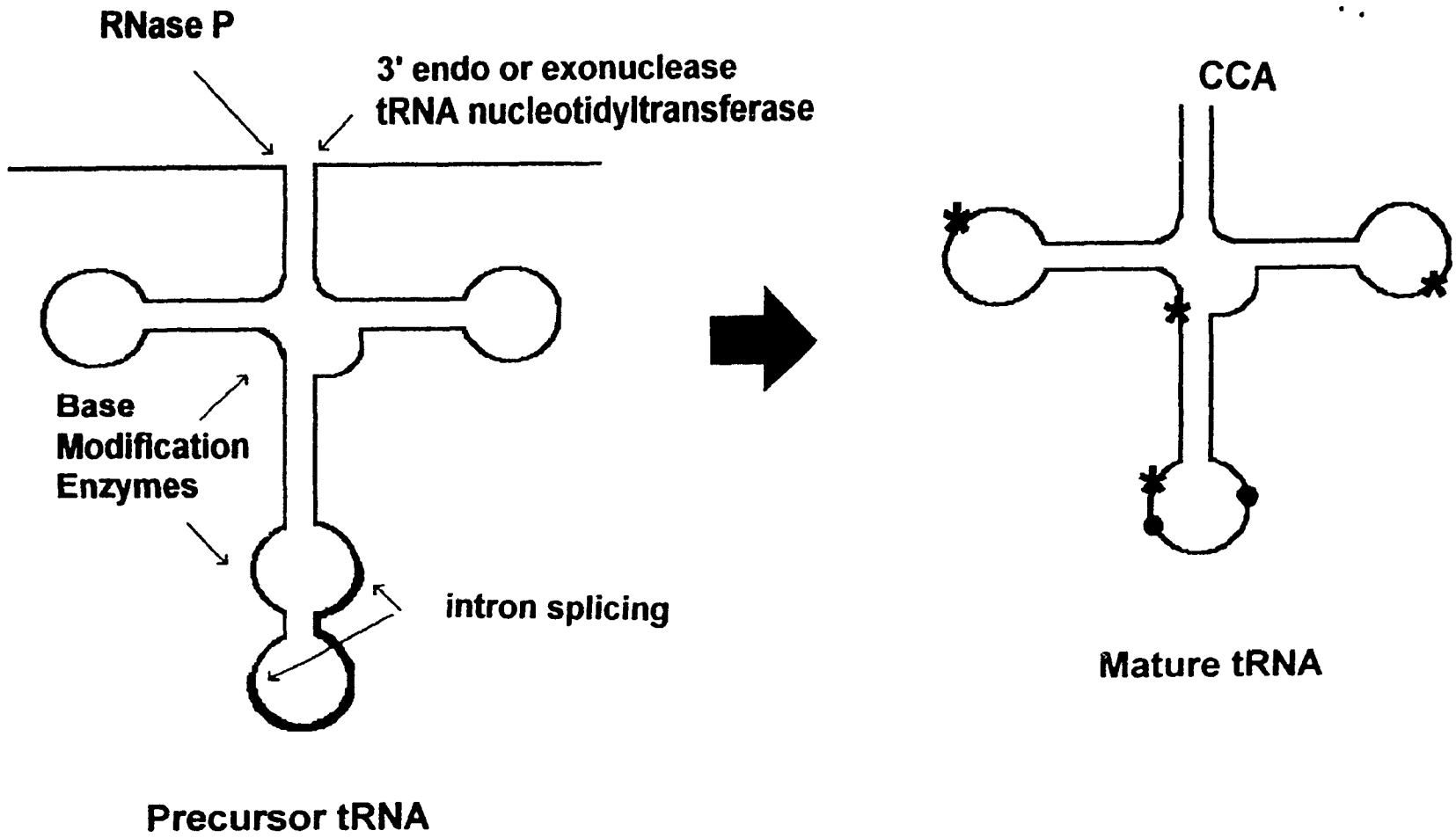
D) Removal of introns

The tRNA precursors in some eukaryotes and archaebacteria as well as in some eubacteria (Moran and Scrimgeour, 1994) require post-transcriptional processing to remove additional nucleotides not only from their 5' and 3' ends but also from within the transcript. These intervening sequences are removed in a process called RNA splicing such that splicing enzymes cleave the introns and the exons are ligated to form a mature tRNA molecule

E) CCA addition

All mature tRNA molecules, both prokaryotic and eukaryotic, possess the sequence CCA at their 3' end. In some prokaryotic tRNA genes such as in *E.coli*, this sequence is encoded in DNA (Komine *et al.*, 1986), but the primary transcripts of other prokaryotic tRNA genes including those of *B. subtilis* (Asha *et al.*,1983) lack the 3' CCA. This sequence is required for tRNA function. If it is not encoded by the gene, the CCA sequence must be added post-transcriptionally after all other types of processing at the 3' end have been completed (Deutscher, 1984). The sequence CCA is added by the action of ATP(CTP): tRNA-specific tRNA nucleotidyltransferase. In *E.coli*, this enzyme also catalyzes replacement of terminal adenylate residues that have been lost due to the activity of RNaseT and other exonucleases (Komine et al., 1986). CCA is added post-transcriptionally to all eukaryotic tRNAs in a similar reaction. A summary of the steps in the nucleolytic processing of eukaryotic tRNA precursors is shown in Figure 2.

FIGURE 2. EUKARYOTIC TRANSFER RNA MATURATION



3. TRANSFER RNA MATURATION OCCURS IN MULTIPLE CELLULAR COMPARTMENTS

Transfer RNA genes are found in the nuclear and mitochondrial genomes of most eukaryotes as well as in the chloroplast in plants. As functional tRNAs are required for protein synthesis, the enzymes required to synthesize mature tRNAs can also be found in these locations.

A) Maturation of mitochondrial tRNA

Some mitochondria use a genetic code that is different from that used by nuclear DNA of the same cell. In order to synthesize the few proteins encoded by mitochondrial DNA, mitochondria possess unique tRNA molecules that recognize the codons of mitochondrial mRNA. Studies using rat liver mitochondria (Manam and Van Tuyle, 1987) and yeast mitochondria (Hollingsworth and Martin, 1986; Chen and Martin, 1988) have shown that in these species, mitochondrial tRNA precursors are processed by 5'- and 3'-endonucleolytic cleavages. Plant mitochondrial tRNA genes, like their nuclear counterparts, do not encode the 3'-terminal - CCA sequence which is added post-transcriptionally by tRNA nucleotidyltransferase (Hanic-Joyce and Gray, 1990). As in tRNAs from other sources, specific nucleotides in plant mitochondria are subject to post-transcriptional modification (Moran and Scrimgeour, 1994). However, most mitochondrial tRNAs contain fewer modified sites than their cytoplasmic counterparts with less variety in their modified bases compared with cytoplasmic tRNAs (Martin, 1995). To date no plant mitochondrial tRNA genes have been shown to contain introns, so maturation of transcripts of these genes does not involve intron excision and splicing events (Moran and Scrimgeour, 1994). Many animal mitochondrial genomes have been sequenced (Wood and Phua, 1996, Kogelnick *et al.*, 1996) as well those of the yeast *Hansenula wingei*

(Sekito *et al.*, 1995) and the plants *Marchantia polymorpha* (Oka *et al.*, 1992) and *Arabidopsis thaliana* (Klein *et al.*, 1994). Analysis of these sequences as well as those of other mitochondrial genomes suggests that the proteins required for mitochondrial tRNA maturation are not synthesized from mitochondrial genes. Instead, as has been shown with yeast, these proteins are encoded by nuclear genes, translated on cytosolic ribosomes and then imported into the mitochondria where they function (Moran and Scrimgeour, 1994).

Some mitochondria do not encode a complete set of tRNA genes so that some mitochondrial tRNAs are transcribed from nuclear genes and subsequently imported into the mitochondrion. Mitochondrial import of tRNAs has been reported in a variety of species including *Saccharomyces cerevisiae* (Martin *et al.*, 1977, 1979), higher plants (Maréchal-Drouard et al., 1988, 1990; Joyce and Gray, 1989), and protozoans including *Tetrahymena* (Chiu *et al.*, 1975; Suyama, 1986) and trypanosomatids (Simpson *et al.*, 1989; Hancock *et al.*, 1992; Lye *et al.*, 1993; Hauser and Schneider, 1995). The extent of mitochondrial import of nucleus-encoded tRNAs varies greatly among different organisms. In yeast, only one cytosolic tRNA is imported (Tarassov and Entelis, 1992), while in trypanosomatids (Simpson *et al.*, 1989; Hancock and Hajduk, 1990, Hauser and Schneider, 1995) all mitochondrial tRNAs must be imported. In higher plants some mitochondrial tRNAs are organelle-encoded whereas others are nucleus-encoded (Maréchal-Drouard et al., 1988).

B) Maturation of chloroplast tRNA

The mechanisms for tRNA processing observed in chloroplasts are similar to the mechanisms described for tRNA processing in plant mitochondria. Studies using artificial transcripts have shown that chloroplast tRNAs are processed by simple 3'- and 5'-endonucleolytic cleavages (Yamaguchi-Shinozaki *et al.*, 1987). Also, tRNA nucleotidyl-transferase, or some similar activity is required for maturation of the 3' end of chloroplast tRNA (Yamaguchi-Shinozaki *et al.*, 1987). In tobacco, it was shown that one third of

tobacco tRNA genes encode the first C of the 3' CCA sequence. However, the second cytidine and the adenosine residues must still be added post-transcriptionally to yield the mature tRNA (Yamaguchi-Shinozaki *et al.*, 1987). This is similar to what occurs during eukaryotic nuclear tRNA maturation.

Plant chloroplast tRNAs have long introns ranging from 325 - 2526 bases (Maréchal-Drouard *et al* ,1993). Intron splicing in chloroplasts is carried out by specific chloroplast factors after 5' and 3' processing (Maréchal-Drouard *et al.*, 1993).

The complete chloroplast genomes of tobacco, rice and *Marchantia polymorpha* have been sequenced (Maréchal-Drouard *et al.*, 1993). None of the genes encoding the enzymes involved in tRNA maturation are encoded in these genomes. This suggests that these enzymes are encoded by nuclear genes, translated on cytosolic ribosomes and imported into the chloroplast where they function.

4. PROTEIN LOCALIZATION

For tRNAs that are encoded by organellar genomes, tRNA precursor processing occurs inside the organelle (Chen *et al.*, 1992), and both mitochondrial (Manam and Van Tuyle, 1987, Hollingsworth *et al.*, 1986, Chen and Martin, 1988, Hanic-Joyce and Gray, 1990) and chloroplast (Gruissem *et al.*, 1983, Greenberg and Hallick, 1986) extracts containing tRNA processing activities have been described. Although mitochondria and chloroplasts are made up of hundreds of different proteins, only a few of these are encoded by their respective organellar DNAs. In fact, more than 90% of mitochondrial proteins (Glick and Schatz, 1991) and approximately 80% of chloroplast proteins (Glick and Schatz, 1991) are encoded by nuclear genes, translated on cytosolic ribosomes and imported into the correct intracellular location. In cases where tRNAs are coded by nuclear genes but imported into mitochondria it is not clear whether complete maturation occurs before, during, or after import into the organelle (Chen *et al.*, 1992).

In yeast, mitochondrial import of tRNA maturation enzymes has been extensively studied. Among the genes that code for tRNA maturation enzymes that have been well characterized are *CCA1* (Chen *et al.*, 1992), *TRM1* (Ellis *et al.*, 1987) and *MOD5* (Martin and Hopper, 1982). The yeast *TRM1* gene encodes N^2,N^2-dimethylguanosine-specific tRNA methyltransferase (Ellis *et al.*, 1987) This enzyme catalyzes the modification of a guanosine residue at position 26 of tRNAs to dimethylguanosine in specific nuclear, mitochondrial and cytosolic tRNAs. Both mitochondrial and nuclear targeting signals have been identified on the *TRM1* gene product. In fact, the *TRM1* gene product is shared by the mitochondrion and the nucleus but does not appear to be present in the cytoplasm (Martin and Hopper, 1994).

The *MOD5* gene encodes another tRNA base modifying enzyme in yeast which functions in the nucleus, cytoplasm and mitochondrion *MOD5* codes for two proteins that differ from each other by the presence or absence of an amino-terminal extension (Gillman *et al.*, 1991; Slusher *et al.*, 1991). One protein, Mod5p-I is produced when translation starts at an AUG representing the first start codon of the open reading frame This protein is located in the mitochondrion and the cytosol. Translation initiation at the second AUG in this message produces the second protein, Mod5p-II, which is located in the cytosol and nucleus (Martin and Hopper, 1994).

I am most interested in the product(s) of the *CCA1* gene. In yeast, tRNA nucleotidyltransferase is encoded by the *CCA1* gene and is localized to the mitochondrion, the nucleus and the cytosol. The *CCA1* gene has 3 in-frame ATGs at the 5' end of its open reading frame (Chen *et al.*, 1990). When a yeast strain carrying a temperature - sensitive mutation in the *CCA1* gene was placed at the non-permissive temperature (37^{o}C) the cells died and CCA addition to both cytosolic and mitochondrial tRNAs was affected (Chen *et al.*, 1992) indicating that the product(s) of this gene were required for CCA addition in both subcellular locations. Furthermore, mutagenesis experiments on the 5' region of this gene to remove the first 9 or 17 amino acids from the gene product showed that these

amino acids are required for growth that requires mitochondrial respiration, but had no effect on growth that required only the cytosolic enzyme (Chen *et al.*, 1992). Based on this observation it was suggested that the first 17 amino acids could function as a mitochondrial import signal These results suggest that there is a single CCA gene that codes for both the mitochondrial and cytosolic tRNA nucleotidyltransferases in yeast. Recently, it has been shown (Wolfe *et al.*, 1994) that translation from the first start codon provides a mitochondrial targeting signal which promotes efficient import into mitochondria, although some enzymatic activity remains in the cytosol and the nucleus. Translation from a downstream start codon eliminates this mitochondrial targeting signal and provides the nuclear and/or cytosolic activity (Wolfe *et al.*, 1994). These results were supported by indirect immunofluorescence which showed that Cca1p-I synthesized from AUG1 is located primarily in mitochondria with minor activity detected in the nucleus, whereas both Cca1p-II and Cca1p-III (synthesized from AUG2 and AUG3 respectively) are located in the nucleus and cytosol (Wolfe *et al*, 1996).

These studies suggest that at least three yeast enzymes involved in tRNA maturation carry targeting signals which allow them to function in multiple cellular compartments. Since my research has concentrated on the purification of one specific tRNA maturation enzyme, tRNA nucleotidyltransferase, I will now concentrate on what is known about this enzyme.

5. TRANSFER RNA NUCLEOTIDYLTRANSFERASE

As previously mentioned, the enzyme ATP (CTP): tRNA nucleotidyltransferase is responsible for the incorporation of CMP and AMP residues into tRNAs that have an incomplete 3'-terminal cytidine, cytidine and adenosine (CCA) sequence. Among tRNA modifying enzymes in *E.coli*, tRNA nucleotidyltransferase is one of the best characterized (Williams and Schofield, 1977). The CCA sequence is encoded by all *E.coli* tRNA genes (Komine *et al*, 1990) so that tRNA nucleotidyltransferase is not essential in this organism

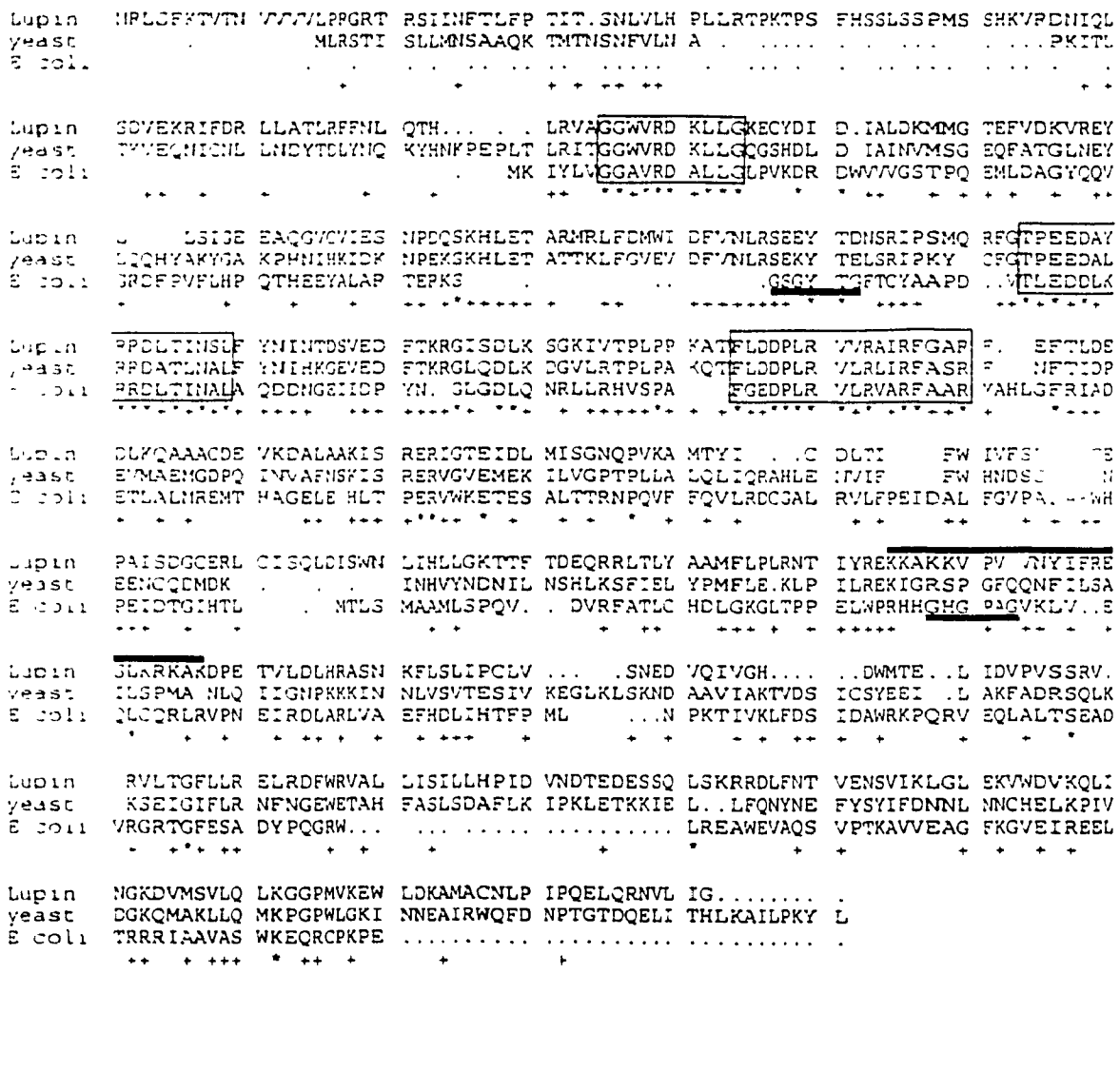
(Zhu and Deutscher, 1986). However, the slow growth rate of *E.coli* tRNA nucleotidyltransferase mutants (Deutscher *et al.*, 1974) suggests a repair function for this enzyme. In contrast, eukaryotic nuclear, mitochondrial and chloroplast tRNA genes lack a complete 3'-terminal CCA (Steinberg *et al.*, 1993) which must be added post-transcriptionally by tRNA nucleotidyltransferase. Therefore, as indicated previously the *CCA1* gene is essential in yeast (Aebi *et al*., 1990). As in *E.coli*, eukaryotic tRNA nucleotidyltransferase also presumably plays a role in repairing damaged 3'-termini

The fact that tRNA nucleotidyltransferase is required in yeast allowed the gene that codes for tRNA nucleotidyltransferase to be isolated by complementation using a yeast genomic library and a temperature-sensitive strain carrying a mutation in the tRNA nucleotidyltransferase gene (Aebi *et al.*, 1990). The amino acid sequence of the yeast tRNA nucleotidyltransferase predicted from the nucleotide sequence of the *CCA1* gene showed similarity to the amino terminal half of the *E.coli* enzyme (Figure 3) In yeast, as described previously, it was shown that a single gene encodes the nuclear, mitochondrial and cytosolic forms of tRNA nucleotidyltransferase (Chen *et al.*, 1992).

In plants active tRNA nucleotidyltransferases must also be found in multiple subcellular locations including the cytosol, nucleus, mitochondrion and chloroplast. We are interested in determining whether the enzymes that function in these different subcellular locations are different proteins encoded by different genes or whether a single gene encodes a tRNA nucleotidyltransferase that functions in more than one intracellular location as is the case in yeast.

Studies of tRNA nucleotidyltransferase have been carried out in eubacteria (Carre *et al.*, 1970, Schofield and Williams, 1977, Leineweber and Philipps, 1978), archaebacteria (Yue *et al.*, 1996) and eukaryotes (Deutscher, 1972, Dullin *et al*., 1975, Cudny *et al*., 1975, Cudny *et al.*, 1978a, Chen *et al.*, 1990, Shanmugam *et al*., 1996).

FIGURE 3. Similarity of the lupin, yeast and *E.coli* tRNA nucleotidyltransferase amino acid sequences



Standard one letter abbreviations are used for the amino acids. * indicates an amino acid conserved in all three sequences. + indicates an amino acid conserved in two of the three sequences. . indicates a gap introduced to optimize alignments. Two putative nucleotide binding domains in the *E.coli* sequence [47] are underlined. Three regions of high amino acid sequence identity are boxed and a potential nuclear localization sequence in the lupin sequence is overlined (Shanmugam *et al.*, 1996)

Specifically, in plants, cytosolic tRNA nucleotidyltransferases have been purified and characterized previously in lupin (Cudny *et al*., 1978a, b, Shanmugam *et al*., 1996) and wheat (Dullin *et al*., 1975). Mitochondrial tRNA nucleotidyltransferases have been partially purified in wheat (Hanic-Joyce and Gray, 1990) and potato (Marchfelder and Brennicke, 1994). However, tRNA nucleotidyltransferases from both the mitochondrion and the cytosol have not been characterized in the same plant at one time. In fact, two forms of this enzyme have only been directly compared in rabbit and rat (Deutscher, 1972a, b, Mukerji and Deutscher, 1972).

Recently, the predicted amino acid sequence of the cDNA encoding the major and presumably cytosolic form of tRNA nucleotidyltransferase from lupin was determined (Shanmugam *et al*., 1996). This work showed that the lupin enzyme has strong predicted amino acid similarity with the yeast enzyme. Like the yeast protein, lupin tRNA nucleotidyltransferase seems to contain additional amino-terminal sequences not present in the *E.coli* protein (Figure 3). These amino-terminal amino acids could potentially encode an organellar targeting signal as has been shown in yeast. It is interesting that within this amino-terminal sequence there is more than one in-frame start codon similar to the situation found in the gene coding for the yeast tRNA nucleotidyltransferase that is targeted to multiple cellular destinations (Martin and Hopper, 1994). It is possible that a situation exists in plants as exists in yeast where a single gene can encode both mitochondrial and nuclear or cytosol forms of the same enzyme. Since plants contain an additional orgenelle, the chloroplast, which contains its own tRNA genes it may also be possible that a single nuclear gene could encode the tRNA nucleotidyltransferase that functions in all of these organelles. There is precedent for the product of a single gene being shared between the mitochondrion and the chloroplast. In pea, glutathione reductase is found in the chloroplast, mitochondrion and cytosol, but is encoded by a single nuclear gene (Creissen *et al*., 1995). While glutathione reductase is not a tRNA processing enzyme, it is the first example of a higher plant protein that is co-targeted to different

organelles (Creissen *et al.*, 1995) and suggests that some plant proteins may be shared between multiple cellular compartments.

6. THIS WORK

The tRNA nucleotidyltransferase enzyme is required for tRNA maturation in multiple cellular compartments including the nucleus, mitochondrion and chloroplast in plant cells. Either all of the cellular forms of this enzyme are the products of a single gene (as in yeast), or multiple genes for tRNA nucleotidyltransferase exist. My research set out to address the question of which of these two models exists in plants. My work concerned plant cells because they serve as a manageable model eukaryotic system and provide an added level of complexity above most other eukaryotes in that they contain an additional organelle, the chloroplast, to which proteins must be directed. Also, although considerable research has been done to determine how nuclear-encoded proteins are targeted to other cellular compartments in fungi and in some animal systems, little is known about how this happens in plants Furthermore, the cytosolic (Chen *et al.*, 1990) and mitochondrial (Chen *et al.*, 1992) forms of tRNA nucleotidyltransferase in yeast show no significant differences for any of the criteria tested. To determine whether or not these cytosolic and mitochondrial forms of tRNA nucleotidyltransferase could be products of a single gene, I isolated tRNA nucleotidyltransferases from the cytosol and mitochondrion of wheat and compared their characteristics.

MATERIALS AND METHODS

1. PROTEIN PURIFICATION

A) Isolation of wheat embryos

Triticum aestivum L. var. Mondor seeds (purchased from Labon Inc.) were milled in a large, commercial Waring blender with a stainless steel container in one kilogram batches (Johnston and Stern, 1957). Seeds were ground for 10 seconds at low speed and half of the ground seeds was transferred to the top of a sieve set consisting of 1.70 mm, 1.18 mm, and 600 μm mesh sizes (top to bottom) and shaken vigorously for 30 seconds on a platform shaker (W.S. Tyler Inc.). Material collected on the top sieve was returned to the Waring blender and the second half of the milled seeds were placed in the top sieve and shaken as above. The contents of the upper sieve were again returned to the blender and ground for 15 seconds. The sieving was repeated a second time as above. All fractions were discarded except for the fraction enriched for embryos on the bottom (600 μm) sieve. This procedure was repeated 1 kg at a time until the embryos from 10 kg of wheat seeds had been collected. Bran was removed from these crude embryos using a hair dryer. Specifically, crude embryos were poured into the 600 μm sieve and cold air from the hair dryer was blown into the sieve while it was agitated manually to remove as much bran as possible. A total of 350 kg of wheat seeds were prepared in this manner in 10 kg lots.

B) Fractionation based on buoyant density

Embryos were separated from other seed particles by fractionation based on buoyant density (Bonen and Gray, 1980). The blown embryos from 10 kg of wheat seeds (with a volume of approximately 200 ml) were placed in a 4 l glass beaker to which 2 l of a cyclohexane/carbon tetrachloride solvent mixture (10/27 v/v) was added. The mixture

was stirred with a glass rod and allowed to stand for a few minutes allowing the embryos to float to the surface, while other seed particles sank. The floating embryos were removed using a strainer and the fractionations repeated twice more using fresh solvent. After the final fractionation, the embryos were poured into a sintered glass funnel and left for 30 minutes under suction. The embryos were then placed in a glass tray in a fume hood and allowed to dry completely for 2 days. After this any remaining bran was removed using the hair dryer method described earlier. At this point the embryos could be stored dry at 4°C until needed.

C) Cell fractionation

Wheat embryos (48 g) from 4°C were distributed in 2.4 g lots on 13.3 cm diameter Petri plates containing a circle of tightly fitting 3MM filter paper that had been moistened with 12 ml of 1% dextrose. The lids were returned and the Petri plates stored in the dark at room temperature for 24 hours before being harvested. From this point all manipulations were performed at 4°C. The germinated embryos were ground with a chilled mortar and pestle in 100 ml of homogenizing buffer (50 mM Tris [pH 8.0], 0.1% w/v BSA, 3 mM EDTA, 0.44 M sucrose, 1 mM β-mercaptoethanol) (Bonen and Gray, 1980) and filtered through 4 layers of cheesecloth The grinding was repeated two more times and the combined filtrates were poured through Miracloth (Calbiochem). The filtrate was divided into four 250 ml Nalgene centrifuge bottles and centrifuged at 1000 X g (2 500 rpm in a Beckman JA-14 rotor) for 10 minutes. The pellet containing nuclei and cell debris was discarded, while the supernatant was centrifuged at 2000 X g (3 500 rpm in a Beckman JA-14 rotor) for 10 minutes. Again the pellet was discarded. The supernatant was centrifuged at 20 000 X g (12 500 rpm in a Beckman JA-20 rotor). The supernatant was used as the cytosolic fraction. The pellets were resuspended in a total of 80 ml of homogenizing buffer using a glass homogenizing tube and Teflon pestle and subjected to

the same series of centrifugations as the first homogenate, i.e, two low speed centrifugations at 1000 X g and 2000 X g in order to remove any nuclei or cellular debris and a high speed centrifugation at 20 000 X g. The pellets of the last high speed centrifugation were resuspended in a total of 16 ml of homogenizing buffer using a glass homogenizer and a Teflon pestle and layered equally onto 6 discontinuous sucrose gradients containing a 12 ml lower layer of 1.55 M sucrose in buffer B (50 mM Tris [pH 8.0], 3 mM EDTA, 1 mM β-mercaptoethanol, 0.1% w/v BSA) (modified from Bonen and Gray, 1980) and a 15 ml upper layer of 1.15 M sucrose in buffer B. The gradients were centrifuged at 40 000 X g for 1 hour, after which the mitochondrial fractions were removed from the 1.15 M and 1.55 M interface by aspiration with a syringe. The mitochondrial fraction was slowly diluted with two volumes of storage buffer (20 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 0.25 M sucrose, 15% (v/v) glycerol and 1 mM β-mercaptoethanol) (Hanic-Joyce and Gray, 1990) and centrifuged at 20 000 X g for 20 minutes. The mitochondrial pellets were resuspended in 1 ml of storage buffer, frozen in liquid nitrogen and stored at -70^{0}C until needed.

D) Preparation of S100 fractions

Frozen mitochondria from 580 g of wheat embryos were thawed on ice and centrifuged at 20 000 X g for 20 minutes. Lysis buffer (20 mM Tris-HCl [pH 8.0], 0.2 mM EDTA, 1 mM β-mercaptoethanol, 1 mM PMSF, 15% v/v glycerol) containing 0.25 M sucrose (Hanic-Joyce and Gray, 1990) at 20 ml for each 4 g of frozen mitochondria was used to resuspend the pellets prior to pelleting again by centrifugation at 20 000 X g for 20 minutes. The pellets were resuspended in 6 ml lysis buffer (without sucrose) and the following were added stepwise with homogenization on ice for 20 strokes in a hand-held homogenizer after each addition: 2.55 ml dH_20; 0.3 ml 20% (v/v) Triton X-100; 3.15 ml 4 M KCl. The lysate was then vortexed for 10 seconds every 5 minutes for 15 minutes

with storage on ice. The lysate was centrifuged at 100 000 X g (23 500 rpm in a Beckman SW-28 rotor) for one hour and the clear amber supernatant (S100) collected. The pellets remaining (P100) were resuspended in 1 ml storage buffer and stored at -70^{O} C.

E) Ammonium sulfate fractionation

Ammonium sulfate fractionation was carried out on both the cytosolic and mitochondrial S100 fractions (Hanic-Joyce and Gray, 1990). The combined S100 fractions from 13 mitochondrial preparations (90 ml) initially were diluted with an equal volume of ammonium sulfate precipitation buffer (90 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 0.5 mM β-mercaptoethanol, 0 5 mM PMSF, 2.5% (v/v) glycerol) (Hanic-Joyce and Gray, 1990) while the cytosolic fraction (500 ml) was used undiluted. In either case the fractions were stirred slowly as 0.1 g/ml of ammonium sulfate was added slowly over 30 minutes. The samples were adjusted to pH 8 with 1M NaOH, left for 30 minutes at 4^{O}C and then centrifuged at 20 000 X g for 20 minutes. The supernatant was collected and three further precipitations were carried out by successive additions of 0.1 g/ml, 0.2 g/ml and 0.2 g/ml of ammonium sulfate as described above. Each of the pellets resulting from ammonium sulfate fractionation of the cytosolic extract was resuspended in 50 ml of dialysis buffer (10 mM Tris-HCl [pH 8], 0.1 mM EDTA, 50 mM KCl, 7.5 % v/v glycerol, 1 mM β-mercaptoethanol, 0 5 PMSF) (Hanic-Joyce and Gray, 1990) while the pellets resulting from the ammonium sulfate fractionation of the mitochondrial S100 extracts were resuspended in 2 ml of dialysis buffer. An aliquot of the supernatant from the final ammonium sulfate fraction was saved to check for residual tRNA nucleotidyltransferase activity.

F) Dialysis

All of the mitochondrial and cytosolic ammonium sulfate fractions (10% w/v, 20% w/v, 40% w/v, and 60% w/v) as well as the >60% w/v supernatants were dialysed for 4 hours at 4°C against 5 l of modified dialysis buffer (Hanic-Joyce and Gray, 1990) in which the salt concentration was reduced from 50 mM KCl to 20 mM KCl. The dialysis was repeated in the same volume of fresh buffer overnight. The dialysates were then stored at -70°C until needed.

G) Protein concentration

Protein concentrations were determined using the BioRad protein assay reagent. The procedure was a modified Bradford (Bradford, 1972) assay as suggested by the manufacturer such that the dye reagent was diluted 1:3 in dH_2O. For each assay 800 µl of the diluted dye reagent was added to a total volume of 200 µl containing sample and dH_2O. Samples that were too concentrated were diluted with dH_2O prior to being assayed. The total assay volume was 1 ml and the protein content was determined by measuring the absorbance at 595 nm on a LKB Novaspec II spectrophotometer (Pharmacia). Protein concentrations were determined by interpolation from a standard curve prepared by measuring the absorbance of different known concentrations (2 µg to 20 µg) of bovine serum albumin. A new standard curve was plotted for each new preparation of Bradford reagent.

H) Measurement of tRNA nucleotidyltransferase activity

The method of Shanmugam *et al.* (1996) was used with some modifications to assay tRNA nucleotidyltransferase activity. Assays were carried out in 0.1 ml volumes and contained 100 mM glycine buffer (pH 9.0), 10 mM $MgCl_2$, 0.2 mM CTP, 0.02 mM ATP, 0.15 µM [α^{32}P] ATP (3000 Ci/mmol, 1mCi/ml), and 20 µg total wheat tRNA type V (Sigma). The amount of protein added to the reaction varied from 2-20 µg during

purification. For characterization of the enzyme, 10 µl of the most purified cytosolic fraction (#121 off affinity column) was used, while 1 µl of the most purified mitochondrial fraction (#124 off affinity column) was used. Each assay was carried out at room temperature for 20 minutes and the reaction stopped by adding 100 µl of cold ($4^{o}C$) 2N HCl. Samples were placed on ice for 30 minutes and filtered through GF/C (Whatmann) glass fiber filters using a Millipore sampling manifold. Each filter was washed twice with 50 ml of 1N HCl and then with 99% ethanol to remove any unincorporated free [$\alpha^{32}P$] ATP. The filters were placed in a $37^{o}C$ incubator until they were dry. The filters were placed in scintillation vials containing 5 ml of scintillation fluid (Cytoscint from ICN) and the counts were measured in a LKB WALLAC - 1218 RACKBETA scintillation counter

I) Column chromatography

Both the mitochondrial and cytosolic 20% - 40% (w/v) ammonium sulfate fractions were chosen for further purification since they contained the most tRNA nucleotidyltransferase activity. All columns were run using a Pharmacia P-1 peristaltic pump and fractions were collected using a Pharmacia Redifrac fraction collector.

i) DEAE Chromatography

The first purification step is based on a modified procedure of Cudny *et al.*, (1978-2). The dialyzed active mitochondrial fraction from ammonium sulfate fractionation was diluted three-fold in modified dialysis buffer and loaded directly at 3 ml/min onto a 1.5 x 10 cm DEAE-Sepharose fast flow column (Pharmacia) which had been packed at 6 ml/min and equilibrated with twenty column volumes of modified dialysis buffer. The column was washed with 150 ml of modified dialysis buffer until protein was no longer detected in the column wash (absorbance of the wash at 595 nm was below 0.1).

Additional proteins including tRNA nucleotidyltransferase were eluted with 250 ml of a 20 - 500 mM KCl linear gradient in modified dialysis buffer Fractions of 2 5 ml were collected and protein concentrations were determined for every third fraction. These fractions were also tested for tRNA nucleotidyltransferase activity as described previously

The dialyzed active cytosolic fraction from ammonium sulfate fractionation was diluted six-fold in modified dialysis buffer and loaded onto a 3 x 40 cm DEAE-Sepharose fast flow column (Pharmacia) that had been equilibrated with modified dialysis buffer Only 21% (0.42 g) of the active 20% - 40% (w/v) ammonium sulfate fraction was loaded to ensure that the column was not overloaded. Subsequent calculations were corrected for this. The column was washed with 300 ml of buffer and proteins eluted with a 400 ml 20 - 500 mM KCl linear gradient in the same manner as the mitochondrial fraction except that 10 ml fractions were collected and assayed

ii) Hydroxylapatite (HA) chromatography

The next purification step was a modified procedure of Cudny et al., (1978a). Active fractions from the DEAE column were pooled and dialyzed against sodium phosphate buffer (10 mM sodium phosphate [pH 7.4] 10 mM $MgCl_2$, 10% glycerol and 5 mM ß-mercaptoethanol) and loaded onto a 1 5 x 4 cm (mitochondrial enzyme) or 3 x 15 cm (cytosolic enzyme) hydroxylapatite column (Pharmacia). The columns were packed at a flow rate of 0.8 ml/minute and equilibrated with twenty column volumes of sodium phosphate buffer (pH 7.4). After absorption of the dialysate to the DEAE column, the column was washed with dialysis buffer (200 ml for the mitochondrial enzyme, 250 ml for the cytosolic enzyme) until the absorption of the fractions at 595 nm was below 0 1 The protein retained on the column was eluted using a 10 - 250 mM sodium phosphate gradient (120 ml for the mitochondrial enzyme, 300 ml for the cytosolic enzyme) at a flow rate of 0.7 ml/min. Fractions, 1.5 ml (mitochondrial enzyme) and 10 ml (cytosolic

enzyme), were collected and every third fraction was assayed for protein content and tRNA nucleotidyltransferase activity.

iii) tRNA-Sepharose affinity chromatography

The final purification step was a modified procedure of Schofield and Williams, (1977). Active fractions from the hydroxylapatite column were pooled and dialyzed against buffer containing 20 mM sodium phosphate (pH 6.0), 10 mM $MgCl_2$, 10% glycerol and 5 mM ß-mercaptoethanol. The dialysate was loaded onto a 1 x 5 cm tRNA-Sepharose affinity column which was kindly provided by Kandavel Shanmugam. The resin was packed at a flow rate of 0.8 ml/min and equilibrated with twenty column volumes of 20 mM sodium phosphate (pH 6.0) buffer. After the active fractions were loaded, the column was washed with 20 mM sodium phosphate buffer (pH 6.0) until neglible protein remained in the column wash. The washing was then continued with 50 mM Tris (pH 8.5) containing 10 mM $MgCl_2$, 5 mM ß-mercaptoethanol and 20% glycerol until the protein content of eluted fractions was below detectable limits. At this point the buffer was changed again to 50 mM Tris (pH 8.5) containing 10 mM $MgCl_2$, 5 mM ß-mercaptoethanol, 200 mM NaCl, 1 mM EDTA and 20% glycerol (Schofield and Williams, 1977). Fractions (1.5 ml) were collected and assayed for protein content and tRNA nucleotidyltransferase activity.

J) Gel filtration chromatography

The native molecular masses of the mitochondrial and cytosolic tRNA nucleotidyltransferases were determined using gel filtration chromatography. Aliquots (200 µl) of the most active fractions off the tRNA affinity column were loaded onto a Superdex^{TM} 75 (Pharmacia) column (1 cm x 30 cm) at a flow rate of 0.2 ml/min and 400 µl fractions were collected. Molecular weight standards used to calibrate the column were

alcohol dehydrogenase (150 kDa), BSA (67 kDa), ovalbumin (43 kDa) and chymotrypsin (25 kDa).

K) SDS polyacrylamide gel electrophoresis

Proteins contained in active fractions were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels consisting of a 10% separating gel and a 4% stacking gel. The gels were made according to the instructions of BioRad and run at constant voltage (200V) for 30 minutes. For each sample 20 µl protein fractions were loaded on the gels. The proteins were visualized by silver staining according to the protocol of the Silver Stain Plus Kit by BioRad.

2. CHARACTERIZATION OF tRNA NUCLEOTIDYLTRANSFERASE

For the detailed characterization of the enzyme, protein from the most active fraction from the tRNA affinity column was used. Fraction 124 was used in the characterization of the mitochondrial enzyme, while fraction 121 was used for the cytosolic enzyme

A) Effect of pH

Standard assay conditions as described in the measurement of tRNA nucleotidyltransferase were used to determine the effects of differing pH on enzyme activity using glycine buffers of pH 7, 7.5, 8, 8.5, 9, 9.5 and 10. The samples were incubated for 20 minutes and the amount of [α^{32}P] AMP incorporated was measured as described previously. The effect of different pHs of Tris buffer was also tested using pH 6, 6.5, 7, 7.5, 8, 8.5 and 9 buffers.

B) Effects of CTP concentration

Using standard activity assay conditions described previously, the effects of varying CTP concentration were studied using a range of CTP concentrations including 0.01 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, 1 mM and 2 mM.

C) Effects of temperature

The effects of temperature on tRNA nucleotidyltransferase activity were studied by measuring the activity of the enzyme over twenty minutes at different incubation temperatures. Temperatures tested were $4^{o}C$, $22^{o}C$, $30^{o}C$, $37^{o}C$, $48^{o}C$, and $72^{o}C$ under standard assay conditions.

D) Ion requirements

The effects on enzyme activity of monovalent (KCl and NaCl) and divalent ($MgCl_2$ and $MnCl_2$) cations were determined using standard assay conditions. Concentrations of 100 mM, 200 mM, 400 mM and 600 mM were tested for KCl and NaCl, while concentrations of 0.1 mM, 1 mM, 10 mM and 100 mM were tested for $MgCl_2$ and $MnCl_2$.

RESULTS

1. PURIFICATION OF tRNA NUCLEOTIDYLTRANSFERASE

A) Cell fractionation

One 48 g sample of wheat embryos was used to determine the distribution of tRNA nucleotidyltransferase in different cellular fractions. The pellet obtained after the initial low speed centrifugation which should contain intact cells and nuclei was designated the nuclear fraction and contained approximately 15% of the total cellular tRNA nucleotidyltransferase activity (Table 1). Most of the activity (80%) was found in the supernatant after high speed centrifugation (Table 1) and was designated the cytosolic fraction. In contrast only about 5% of the total activity (Table 1) was found in the mitochondrial fraction (the pellet obtained from the high speed centrifugation). Similarly, most of the total protein (86%) was found in the cytosolic fraction, while 11% and 3% were recovered in the nuclear and mitochondrial fractions respectively (Table 1) Both the mitochondrial and cytosolic fractions were used for further purification. During the preparation of tRNA nucleotidyltransferase from the 48 g sample of wheat embryos, the distribution of tRNA nucleotidyltransferase in the soluble and membrane-bound fractions of the mitochondria was determined (Table 2). Although enzyme activity was seen in the mitochondrial fraction before lysis, lysis of the mitochondria resulted in a two-fold increase in the total tRNA nucleotidyltransferase activity and a 50% increase in specific activity (Table 2). After centrifugation of lyzed mitochondria at 100 000 X g for 1 hour more than 90% of the tRNA nucleotidyltransferase activity recovered was found in the soluble fraction with only a small amount remaining in the pellet (Table 2). The soluble fractions from 14 mitochondrial preparations were collected and combined for future analysis.

TABLE 1. Distribution of tRNA nucleotidyltransferase by differential centrifugation using 48 g of wheat embryos.

Cellular fraction	Total protein (g)	% Total protein recovered	Total activity (10^6 cpm)	Specific activity (10^6 cpm/g)	% Total activity recovered
Nuclei[1]	0.6	11	220	367	14.5
Cytosol[2]	4.65	86	1210	260	80
Mitochondria[3]	0.17	3	84	494	5.5

[1]Pellet obtained after first low speed centrifugation.
[2]Supernatant after first high speed centrifugation.
[3]Mitochondria obtained after second high speed centrifugation.

TABLE 2. Distribution of tRNA nucleotidyltransferase after fractionation of mitochondria from 48 g of wheat embryos.

Cellular fraction	Protein content (mg)	Activity (10^6 cpm)	Specific activity (10^6 cpm/mg)	% Fraction of total activity	% Fraction of activity recovered after centrifugation (100 000 X g)
Intact mitochondria	67	65	0.97	50	N.A.[1]
Lyzed mitochondria	93	130	1.4	100	N.A.
S100[2]	73	87	1.2	67	92
P100[3]	8	8	1	6	8

[1]N.A.: not applicable.
[2]S100: supernate after centrifugation at 100 000 X g for 1 h.
[3]P100: pellet resulting from centrifugation at 100 000 X g for 1 h.

B) Ammonium sulfate fractionation

i) Mitochondrial protein

Ammonium sulfate fractionation of the soluble fraction from the mitochondrial preparations showed that less than 1% of the tRNA nucleotidyltransferase activity was found in the initial 0-10% (w/v) ammonium sulfate fraction or in the supernatant that remained after 60% (w/v) ammonium sulfate fractionation (Table 3). The majority of the tRNA nucleotidyltransferase activity (71%) was seen in the 20-40% (w/v) fraction with smaller amounts (19% and 8%) found in the 10-20% (w/v) and 40-60% (w/v) fractions, respectively (Table 3). The 20-40% (w/v) ammonium sulfate fraction which showed an approximately 3-fold increase in specific activity was used in subsequent purification steps.

ii) Cytosolic protein

Ammonium sulfate fractionation of the cytosolic extract also showed that most of the tRNA nucleotidyltransferase activity (80%) was found in the 20-40% (w/v) ammonium sulfate fraction with only minor amounts found in any other fraction (Table 3). Again, since this 20-40% (w/v) ammonium sulfate fraction contained the majority of the tRNA nucleotidyltransferase activity and showed an approximately 2-fold increase in specific activity it was used in subsequent purification steps.

C) DEAE column chromatography

i) Mitochondrial protein

DEAE column chromatography was chosen as the next purification step as it had been shown to be useful in the purification of tRNA nucleotidyltransferase from other sources (Cudny *et al*, 1978a; Shanmugam *et al*, 1996). After dialysis, the 20-40% (w/v) ammonium sulfate fraction was loaded onto the column and the column washed until protein levels in the column wash were below the level of detection. At that point a 20 -

TABLE 3. Distribution of tRNA nucleotidyltransferase activity in ammonium sulfate fractions.

	Mitochondrial enzyme				Cytosolic enzyme			
Fraction	Protein content (mg)	Total activity (10^6 cpm)	Specific activity (10^6 cpm/mg)	Fraction of total activity (%)	Protein content (mg)	Total activity (10^6 cpm)	Specific activity (10^6 cpm/mg)	Fraction of total activity (%)
Total	718	640	0.89	100	4750	1500	0.316	100
0 - 10% Ammonium sulfate	5	3.9	0.78	0.6	125	9.4	0.08	0.6
10 - 20% Ammonium sulfate	389	120	0.31	18.8	416	50	0.12	3.3
20 - 40% Ammonium sulfate	182	450	2.5	71	1980	1200	0.606	80
40 - 60% Ammonium sulfate	52	53	1.0	8.4	900	37	0.04	2.5
> 60% Ammonium sulfate	13.5	4.5	0.33	0.7	200	0	0	0

500 mM KCl linear gradient was used to elute proteins from the column (Figure 4). Although 30% of the proteins in the extract did not bind to the resin and were eluted in the buffer wash, more than 85% of the tRNA nucleotidyltransferase activity was eluted between 100-250 mM KCl (Figure 4). Conductivity measurements of the pooled active fractions from the DEAE column showed a KCl concentration of 190 mM (data not shown). The specific activity of the pooled active fractions from the DEAE column was approximately 6.7 X 10^6 cpm/mg protein which represents approximately a 3-fold enrichment at this step (Table 4).

ii) Cytosolic protein

The 20-40% (w/v) ammonium sulfate fraction containing the cytosolic enzyme was dialyzed and loaded onto a DEAE column. Again a 20-500 mM KCl linear gradient was started after the column had been washed such that no protein was detected in the column wash. Approximately 52% of the total protein loaded onto the column was eluted in the wash, although more than 90% of the tRNA nucleotidyltransferase activity bound to the column and was eluted between 150 and 250 mM KCl (Figure 5). Conductivity measurements indicated a KCl concentration of approximately 200 mM for the pooled active fractions (data not shown). The specific activity achieved by DEAE chromatography was 0.69 X 10^6 cpm/mg protein (Table 5) with a major loss of tRNA nucleotidyltransferase activity at this step.

D) Hydroxylapatite column chromatography

i) Mitochondrial protein

Fractions 65-90 off the DEAE column were pooled, dialyzed and loaded onto a hydroxylapatite column. The column was washed with buffer until the protein content was below detectable limits and proteins retained on the column were eluted using a 10-250

FIGURE 4. DEAE-Sepharose chromatography
Mitochondrial tRNA nucleotidyltransferase

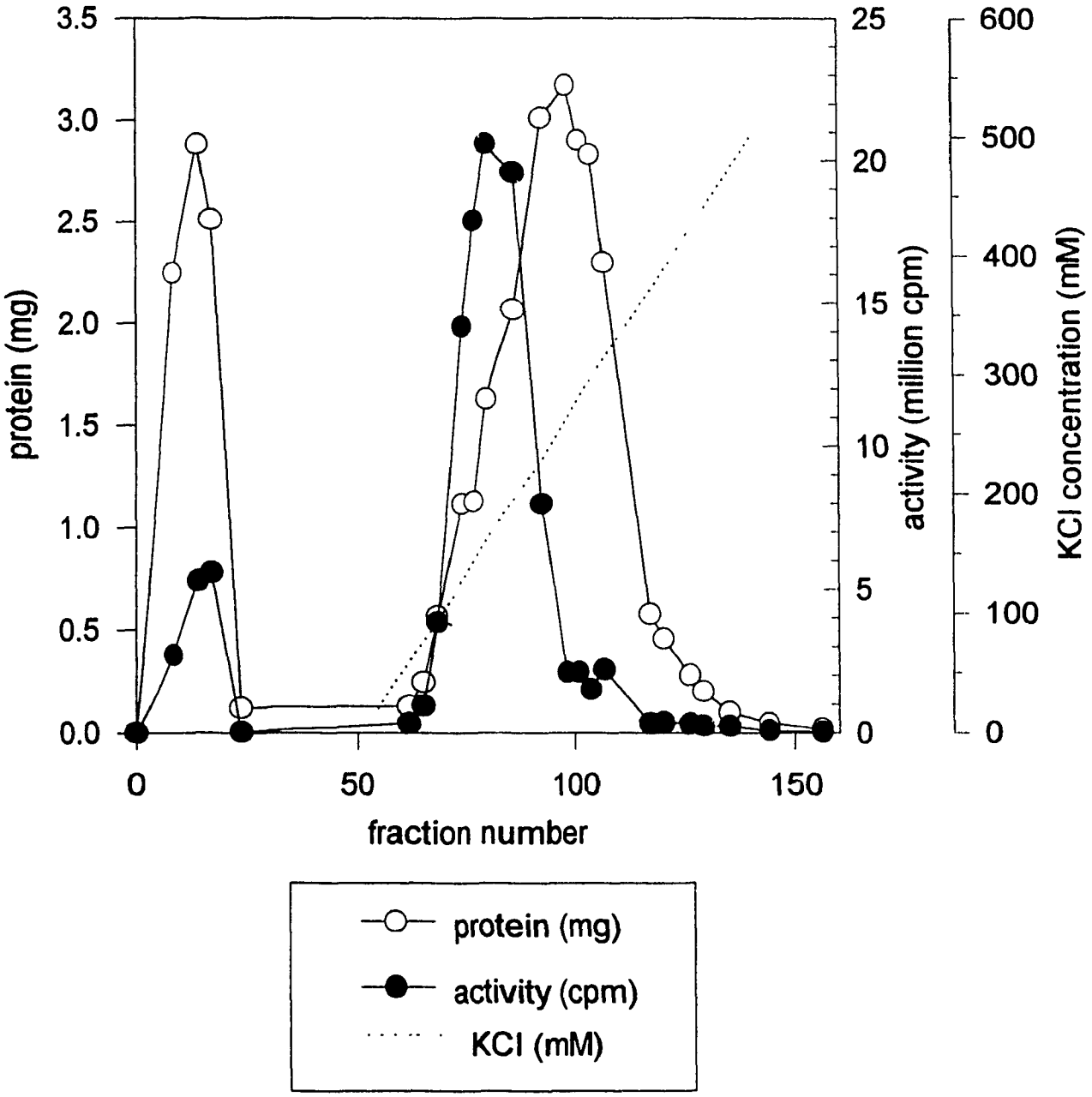


TABLE 4. Purification of wheat mitochondrial ATP (CTP): tRNA nucleotidyltransferase.

Purification step	Protein content (mg)	Total activity (10^6 cpm)	Specific activity (10^6 cpm/mg)	Fold purification	% Recovery
Crude extract	718	640	0.89	1	100
20 - 40% Ammonium sulfate fraction	182	450	2.5	2.8	70
DEAE Column Chromatography	30	200	6.7	7.5	31
Hydroxylapatite Chromatography	4.2	120	28.6	32	19
tRNA Affinity Chromatography	N.D.[1]	33	N.D.	N.D.	5.1

[1]N.D. indicates that this value could not be determined under the conditions used.

FIGURE 5. DEAE-Sepharose chromatography
Cytosolic tRNA nucleotidyltransferase

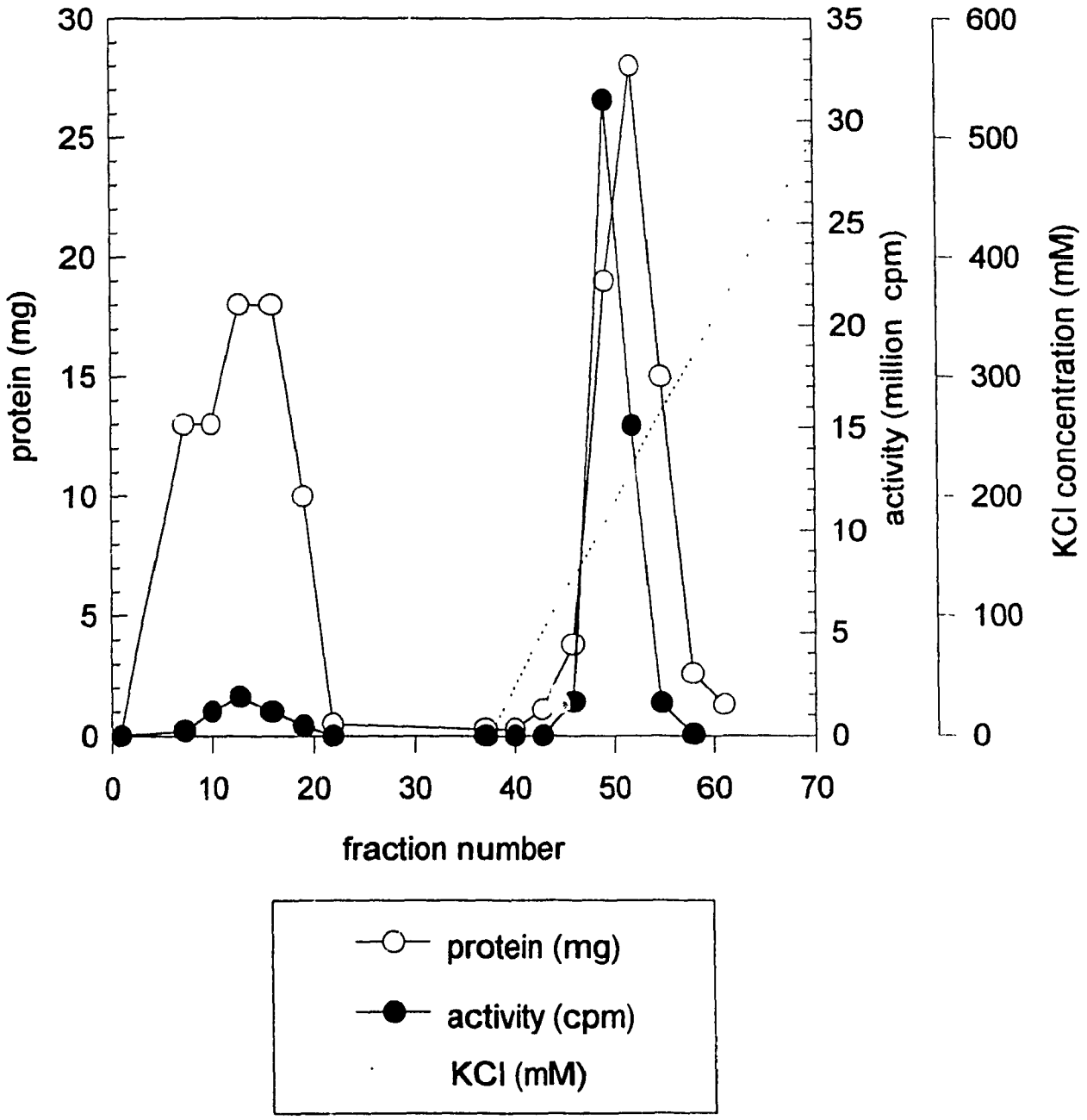


TABLE 5. Purification of wheat cytosolic ATP (CTP): tRNA nucleotidyltransferase.

Purification step	Protein content (mg)	Total activity (10^6 cpm)	Specific activity (10^6 cpm/mg)	Fold purification	% Recovery
Crude extract	4750	1500	0.32	1	100
20 - 40% Ammonium sulfate fraction	1980	1200	0.6	1.9	80
DEAE Column Chromatography	424	268	0.69	2.2	19
Hydroxylapatite Chromatography	62	71	1.15	3.6	4.8
tRNA Affinity Chromatography	0.1	3.62	36.2	113	0.24

mM linear phosphate buffer gradient. While a sizeable amount of protein (67%) was eluted in the column wash, it was devoid of any tRNA nucleotidyltransferase activity (Figure 6). The tRNA nucleotidyltransferase was eluted between 75-150 mM sodium phosphate (Figure 6). The sodium phosphate concentration of the pooled active fractions was determined to be 110 mM by conductivity measurements (data not shown). The specific activity of the pooled active fractions was 28.6 X 10^6 cpm/mg protein (Table 4) which represents an increase of approximately 4-fold in this step.

ii) Cytosolic protein

Active fractions off the DEAE (fractions 46-52) column were pooled, dialyzed and loaded onto an hydroxylapatite column. As with the mitochondrial enzyme little tRNA nucleotidyltransferase activity was found in the column wash (Figure 7) The majority of tRNA nucleotidyltransferase activity eluted from the column at a range of 150-240 mM sodium phosphate, although approximately 20% of the tRNA nucleotidyltransferase activity was eluted from this column with approximately 80 mM sodium phosphate Conductivity measurements showed that the sodium phosphate concentration of the pooled active fractions in the major peak of tRNA nucleotidyltransferase was 200 mM (data not shown). The specific activity of the pooled active fractions (fractions 50-58) was 1.15 X 10^6 cpm/mg protein (Table 5) and this step showed a 2-fold increase in specific activity.

E) tRNA affinity column chromatography

i) Mitochondrial protein

Active fractions from the hydroxylapatite column were pooled, dialyzed and loaded onto a tRNA affinity column which was washed until protein levels dropped to a negligible

FIGURE 6. Hydroxylapatite chromatography
Mitochondrial tRNA nucleotidyltransferase

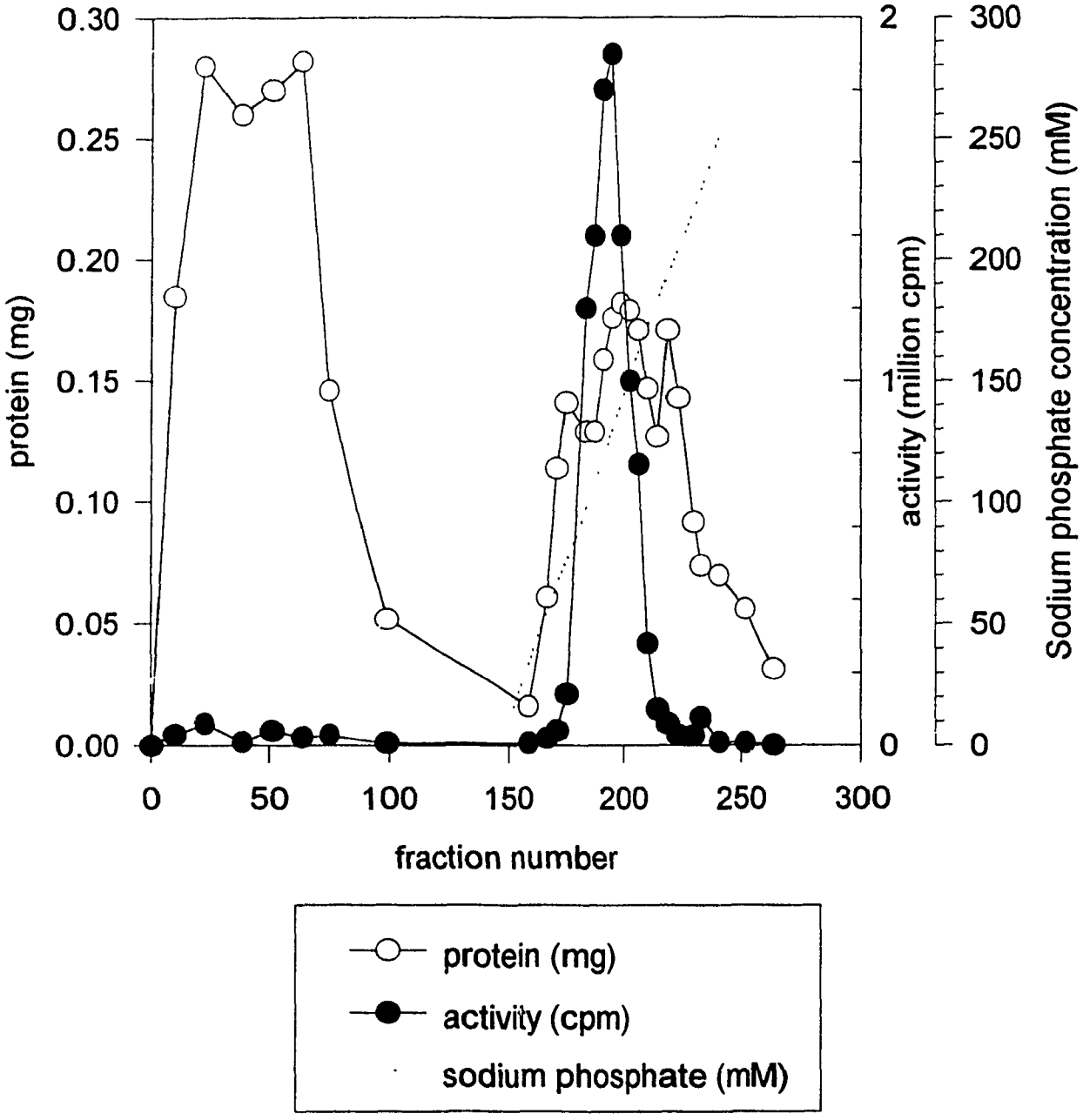
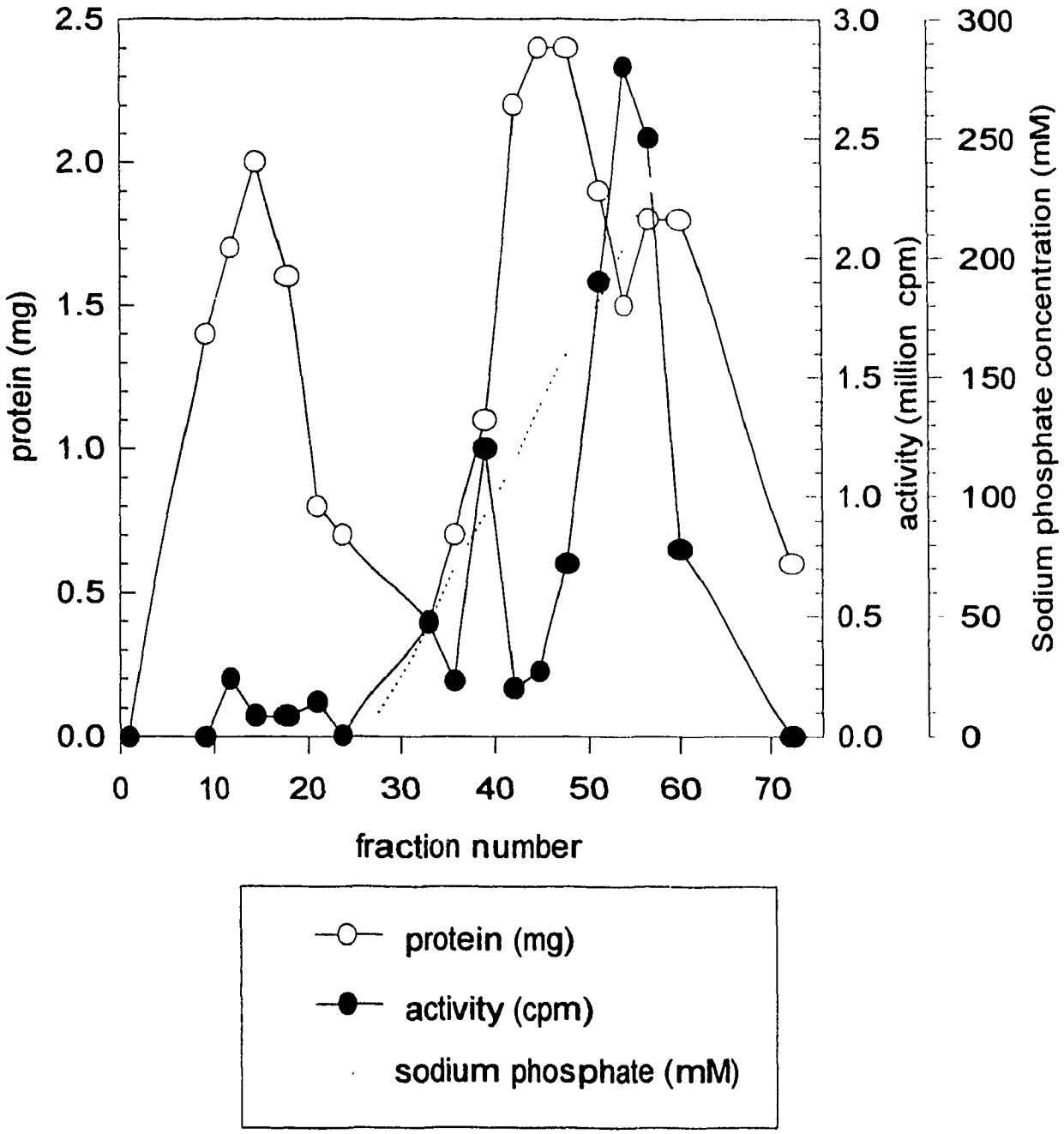


FIGURE 7. Hydroxylapatite chromatography
Cytosolic tRNA nucleotidyltransferase



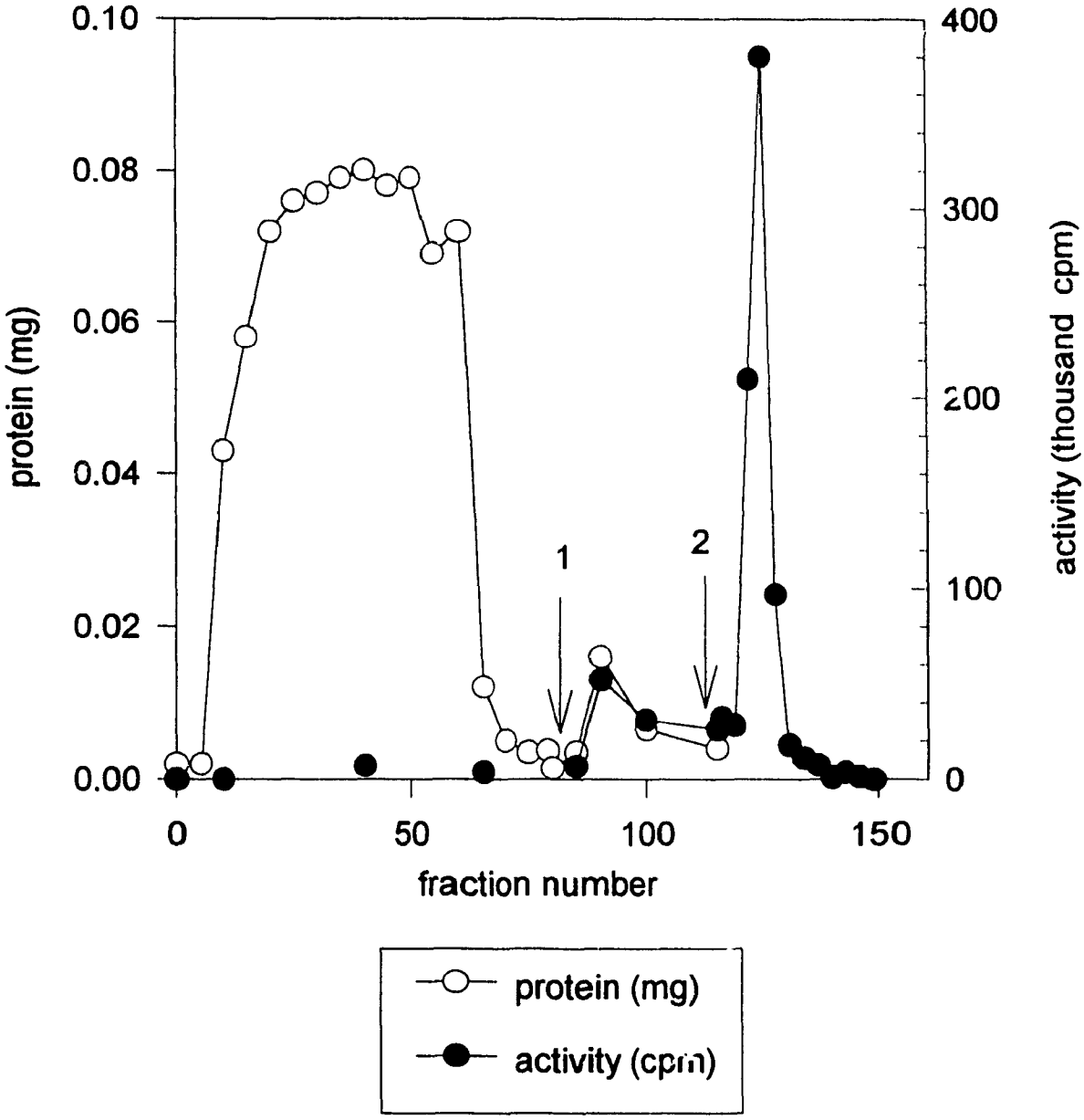
level (Figure 8). At this point (fraction 80) washing was continued with 50 mM Tris-HCl (pH 8.5) until protein levels were negligible. At this point (fraction 116) protein was eluted with the addition of buffer containing 200 mM NaCl and 0.1 mM EDTA. Although most of the protein (94%) was eluted with the column wash with some also eluting when the buffer was changed to Tris-HCl (pH 8.5), the majority of the tRNA nucleotidyltransferase activity was eluted in the first few fractions after buffer containing 200 mM NaCl and 1 mM EDTA was added (Figure 8). At this point the protein in the active fractions was below the level of detection of the Biorad assay, so that a precise specific activity for these fractions could not be calculated. However, using the fact that the lower detection limit of the protein assay is 1 μg of protein, a minimum 370-fold enrichment in activity was achieved although 75% of the remaining activity is lost at this step. Starting with a crude mitochondrial extract from 570 g of wheat embryos a 5% yield of activity was achieved (Table 4).

ii) Cytosolic protein

Fractions 50-58, defining the major peak of tRNA nucleotidyltransferase activity from the HA column, were pooled, dialyzed and loaded onto a tRNA affinity column. As was seen with the mitochondrial enzyme little protein (6%) was retained on the column and almost none of the tRNA nucleotidyltransferase was found in the column wash (Figure 9). Instead, most of the enzyme activity (99%) was eluted in the first few fractions after the addition of buffer containing 200 mM NaCl and 1mM EDTA. Starting from 48 g of wheat embryos only an 113-fold enrichment of tRNA nucleotidyltransferase activity was achieved with only about 0.2% of activity recovered with respect to the crude extract (Table 5).

FIGURE 8. tRNA Affinity chromatography

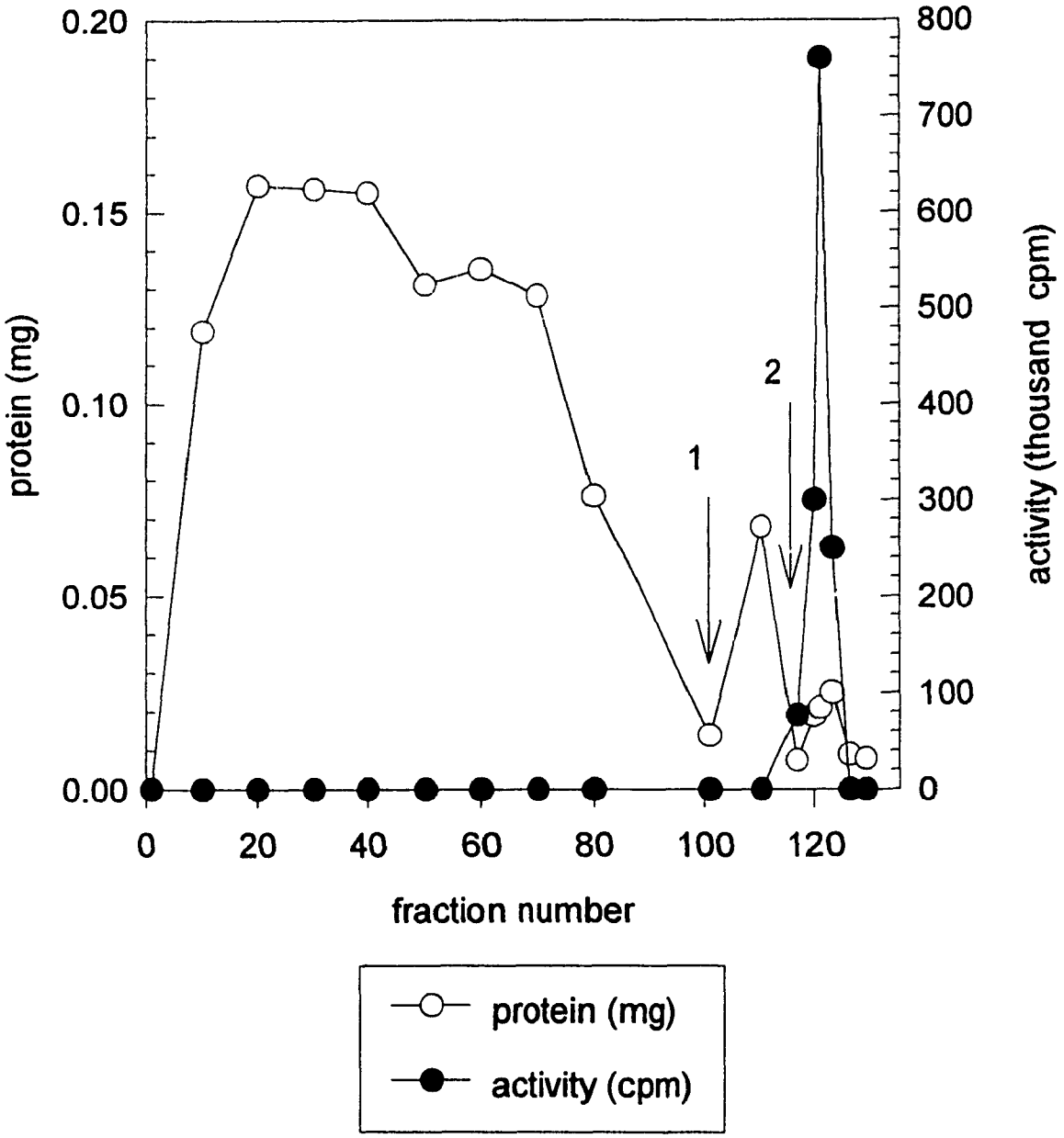
Mitochondrial tRNA nucleotidyltransferase



1: Addition of Tris-HCl buffer (pH 8.5)
2: Addition of 200 mM NaCl and 0.1 mM EDTA

FIGURE 9. tRNA Affinity chromatography

Cytosolic tRNA nucleotidyltransferase



1: Addition of Tris-HCl buffer (pH 8.5)

2: Addition of 200 mM NaCl and 0.1 mM EDTA

F) SDS-polyacrylamide gel electrophoresis

i) Mitochondrial protein

Proteins contained in the active fractions were resolved by SDS-polyacrylamide gel electrophoresis and visualized by silver staining (Figure 10). The fraction showing highest activity obtained from the final purification step showed two major protein bands at approximately 60 KDa (Figure 10, lane 7). This apparent molecular mass was confirmed by loading an aliquot of the most active fraction from the tRNA affinity column (#124) onto a Superdex^{TM} 75 gel filtration column. A molecular mass of 60 kDa was assigned to the fraction showing maximum activity based on the elution of protein markers of known size (Figure 11).

ii) Cytosolic protein

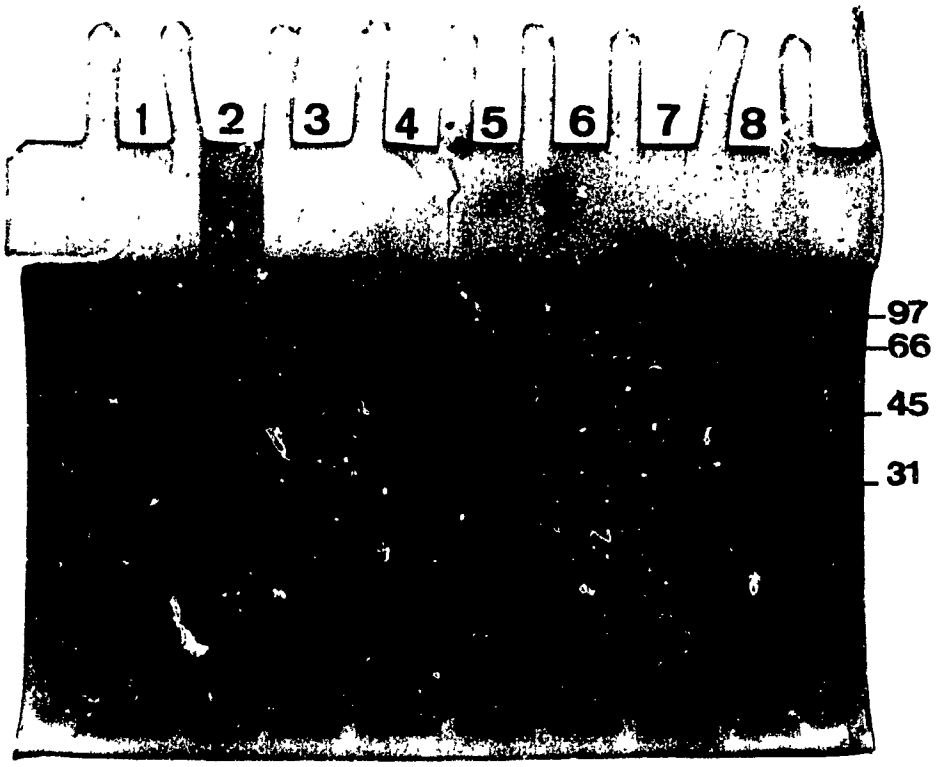
Proteins contained in each step of the affinity column (including the most active fraction) were resolved by SDS-polyacrylamide gel electrophoresis and electrophoresed along with their mitochondrial counterparts (Figure 12). The most active cytosolic fraction shows three major bands at approximately 66 KDa which are also present in the column wash (compare lanes 2 and 4). An aliquot of the most active fraction from the tRNA affinity column (#121) was loaded onto a Superdex^{TM} 75 gel filtration column. Maximum activity was observed at 66 KDa molecular mass (Figure 11) based on the elution of protein markers of known size.

2. CHARACTERIZATION OF tRNA NUCLEOTIDYLTRANSFERASE

The effects on enzyme activity of different general conditions were tested in order to see whether the mitochondrial and cytosolic tRNA nucleotidyltransferases shared similar

FIGURE 10.

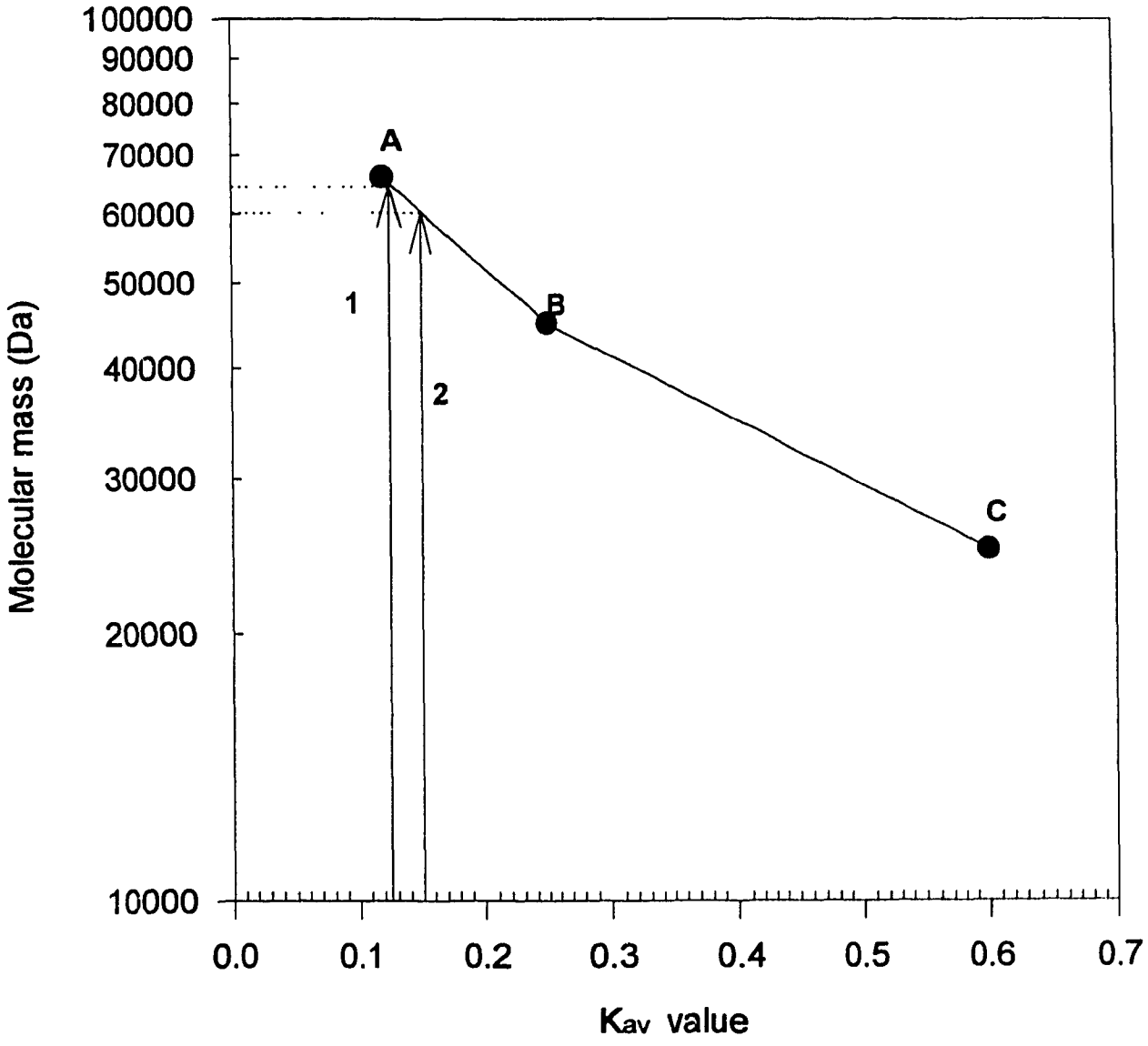
SDS-PAGE ANALYSIS OF THE PURIFICATION OF MITOCHONDRIAL tRNA NUCLEOTIDYLTRANSFERASE



Lane
1 BioRad low range molecular weight markers
2 20-40% ammonium sulfate fraction
3 pooled, active DEAE fractions
4 pooled, active HA fractions
5 flowthrough off tRNA affinity column
6 fraction with low activity off tRNA affinity column
7 fraction with highest activity off tRNA affinity column
8 BioRad low range molecular weight markers

FIGURE 11.

Molecular mass determinations for cytosolic and mitochondrial tRNA nucleotidyltransferases from gel filtration chromatography

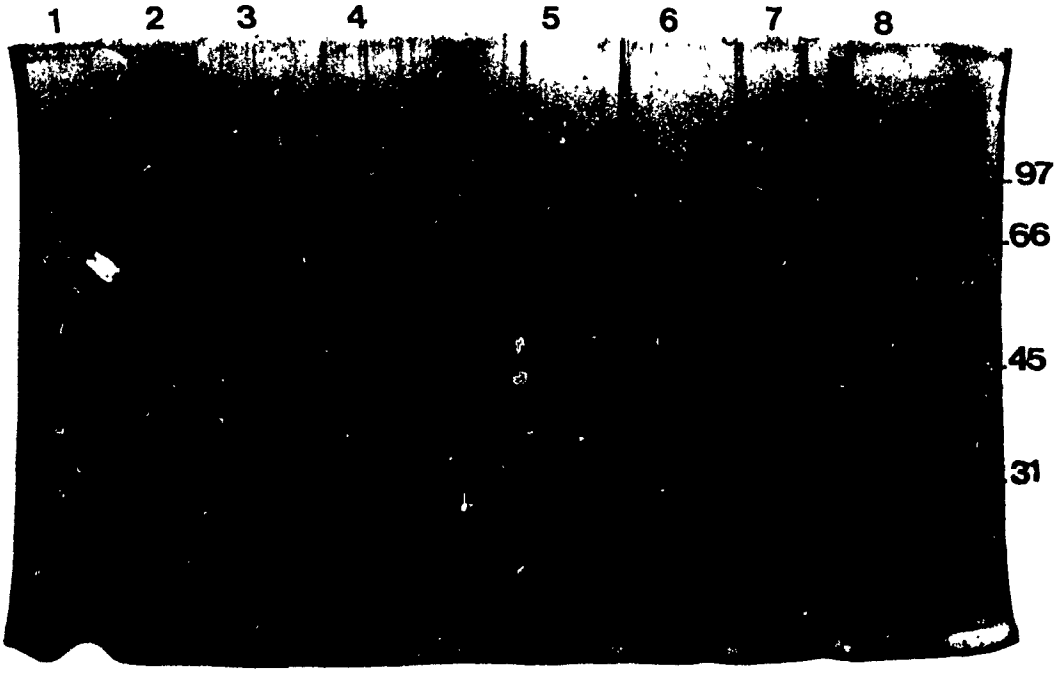


molecular mass standards :
A= BSA, 67 kDa
B= ovalbumin, 45 kDa
C= chymotrypsin, 25 kDa

active fraction from gel fitration column :
1= cytosolic enzyme
2= mitochondrial enzyme

FIGURE 12.

SDS-PAGE ANALYSIS COMPARING THE MITOCHONDRIAL AND CYTOSOLIC PROTEINS



Lane 1 BioRad low range molecular weight markers
2 cytosolic fraction (flowthrough) with low activity off affinity column
3 cytosolic fraction with low activity off affinity column
4 cytosolic fraction with highest activity off affinity column
5 mitochondrial fraction with highest activity off affinity column
6 mitochondrial fraction with low activity off affinity column
7 mitochondrial fraction (flowthrough) with low activity off affinity column
8 BioRad low range molecular weight markers

enzymatic characteristics. The most active fractions from the affinity columns were used for these assays, specifically fraction 124 for the mitochondrial protein (Figure 8) and fraction 121 for the cytosolic protein (Figure 9). All experiments were performed at least twice and with samples in duplicate. Initial experiments performed to determine the temperature optimum under standard assay conditions showed maximum AMP incorporation at 37°C. However, a range of temperatures extending from 22°C to 48°C showed high levels of activity (data not shown), therefore, for convenience 22°C was chosen as the incubation temperature for all subsequent experiments.

A) pH optimum

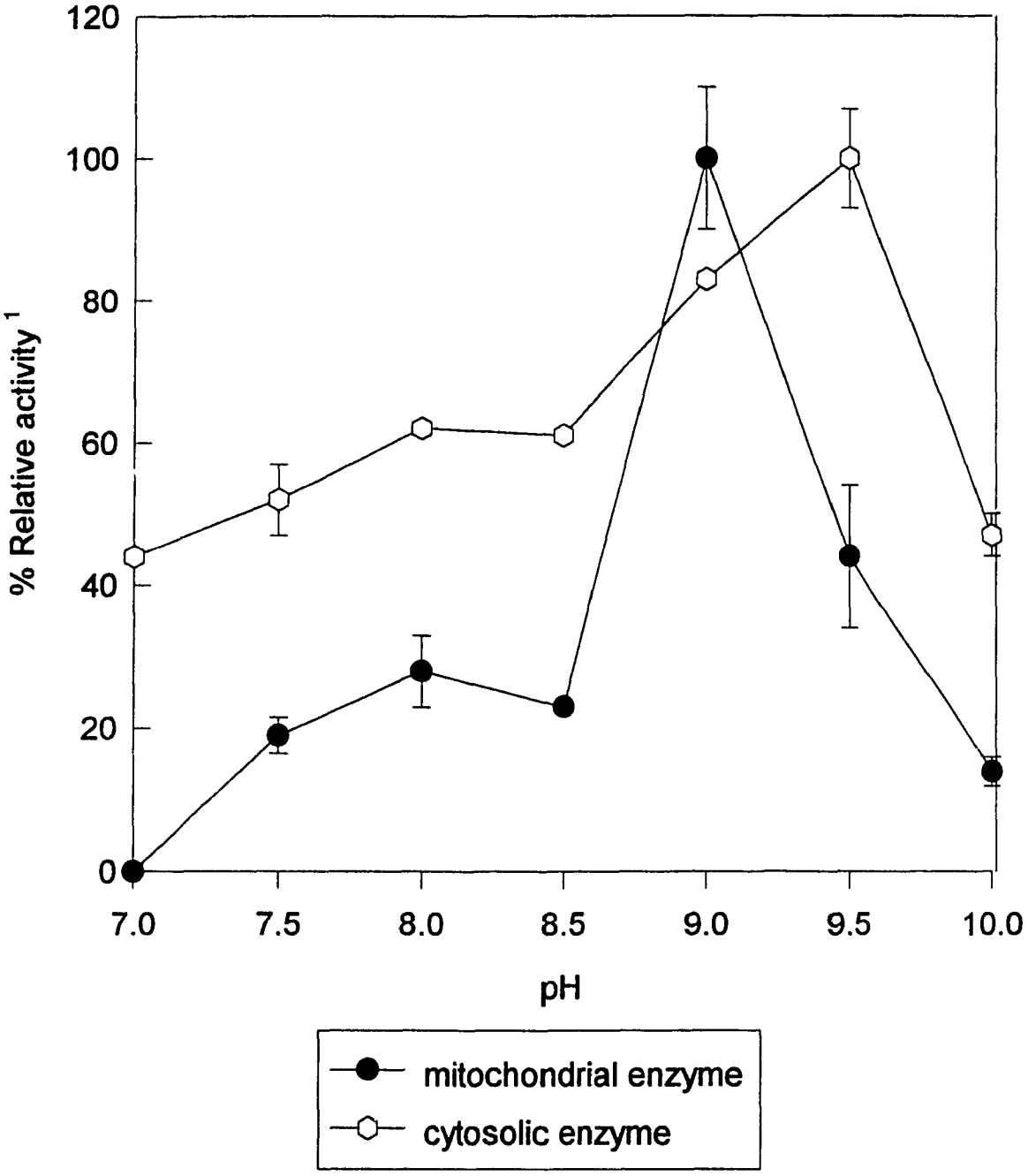
The effect of altering pH on mitochondrial and cytosolic tRNA nucleotidyltransferase activity was tested. Standard assay conditions as described in the experimental section were used except that the pH of the 100 mM glycine buffer was changed to pH 7.5, 8, 8.5, 9, 9.5 or 10 Both the mitochondrial and cytosolic tRNA nucleotidyltransferases showed maximum activity near pH 9 (Figure 13), although the mitochondrial protein seems to have a pH optimum at 9 in glycine buffer, while the cytosolic optimum occurs at pH 9.5.

The effect of altering pH on enzyme activity was also tes ed in Tris-HCl buffer at pH 6, 6.5, 7, 7.5, 8, 8.5 and 9. Both the cytosolic and mitochondrial enzymes showed a biphasic activity profile. The mitochondrial protein showed greatest activity at pH 7 and 9 (Figure 14) while the cytosolic protein showed greatest activity at pH 7.5 and 9.

B) Requirements for metal ions

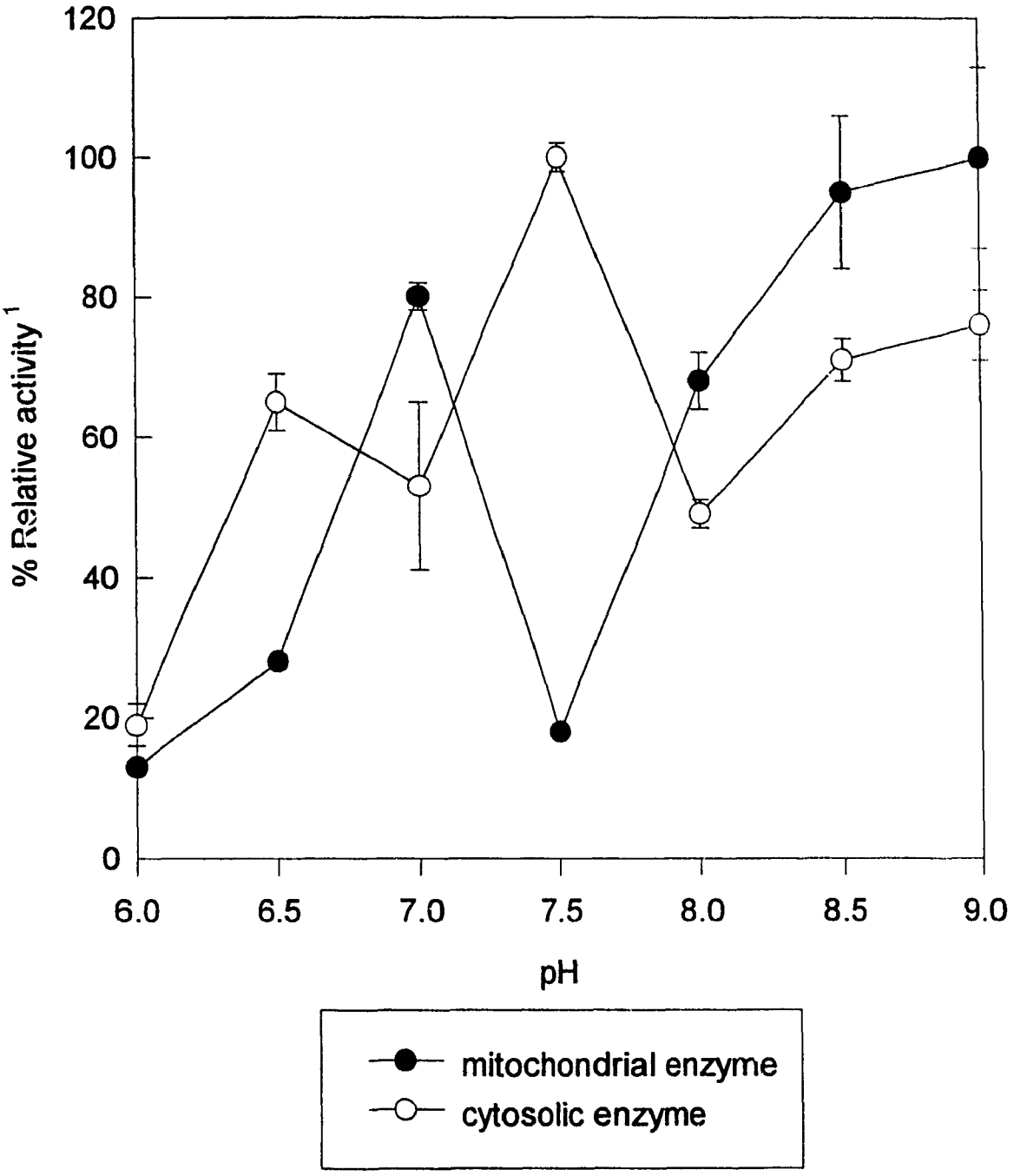
Using standard assay conditions the effects on enzyme activity of varying amounts of metal ions were studied. Different amounts of NaCl and KCl (Table 6) or $MgCl_2$ and

FIGURE 13. Effect of altering pH of glycine buffer on tRNA nucleotidyltransferase activity



[1] Relative activity for each enzyme is the ratio of AMP incorporated at a specific pH compared to the maximum incorporation of AMP achieved for that enzyme (expressed as a percentage).
Error bars represent the average difference in AMP incorporation between two samples in two experiments.

FIGURE 14. Effect of altering pH of Tris-HCl buffer on tRNA nucleotidyltransferase activity



[1]Relative activity for each enzyme is the ratio of AMP incorporated at a specific pH compared to the maximum incorporation of AMP achieved for that enzyme (expressed as a percentage).
Error bars represent the average difference in AMP incorporation between two samples in two experiments.

TABLE 6. Effect on tRNA nucleotidyltransferase activity of altering NaCl and KCl concentration.

Relative activity (%)[1]				
	Mitochondrial enzyme		Cytoplasmic enzyme	
Ion concentration (mM)	NaCl	KCl	NaCl	KCl
0[2]	100	100	100	100
100	59	99	66	82
200	40	77	46	27
400	43	33	8	22
600	36	59	6	26

[1]For each cation relative activity for each enzyme is the ratio of incorporation of AMP achieved under the specific experimental conditions compared to the maximum incorporation of AMP achieved for that cation (expressed as a percentage).

[2]Each mitochondrial enzyme assay contained 0.8 mM NaCl which was added as part of the enzyme fraction. Each cytoplasmic assay contained 8 mM NaCl which was added as part of the enzyme fraction.

$MnCl_2$ (Table 7) were added to the reaction mixtures and enzyme activity measured. Highest activity for both the cytosolic and mitochondrial enzymes was seen with no addition of NaCl or KCl added. These reactions, however, contained 0.8 mM NaCl (mitochondrial enzyme) or 8 mM NaCl (cytosolic enzyme) which was added with the enzyme extract. For both the mitochondrial and cytosolic enzymes 100 mM NaCl inhibited activity to a greater extent than did 100 mM KCl (Table 6). By 200 mM salt concentration both the mitochondrial and cytosolic enzymes showed a large degree of inhibition with the degree of inhibition at salt concentrations greater than 200 mM more pronounced with the cytosolic protein than with the mitochondrial enzyme (Table 6).

In addition to the $MgCl_2$ that was added to each assay, each sample also contained 0.08 mM $MgCl_2$ (mitochondrial enzyme) or 0.8 mM $MgCl_2$ (cytosolic enzyme) from the enzyme extract added. Both the mitochondrial and cytosolic enzymes showed maximal activity when the $MgCl_2$ or $MnCl_2$ concentrations were 10 mM (Table 7). While activity increased with increasing $MgCl_2$ concentrations up to 10 mM, at 100 mM $MgCl_2$ concentration activity had been reduced by 50% for the mitochondrial enzyme and 80% for the cytosolic enzyme. When the $MnCl_2$ concentration was increased from 1 mM to 10 mM, the cytosolic enzyme showed a 97% increase in activity while the mitochondrial enzyme showed only a 25% increase in activity. By comparison, increasing the $MgCl_2$ concentration from 1 mM to 10 mM resulted in a 23% increase in activity in the mitochondrial enzyme and a 16% increase in activity for the cytosolic enzyme.

C) Effect of CTP concentration on enzyme activity

The effect of altering CTP concentration on enzyme activity was tested. Standard assay conditions were used as previously described except that the amounts of CTP used were varied (0 mM, 0.01 mM, 0.1 mM, 0.2 mM, 0 4 mM, 0 8 mM, 1 mM and 2 mM). A gradual increase in the incorporation of [α^{32}P] ATP was seen for both the mitochondrial

TABLE 7. Effect on tRNA nucleotidyltransferase activity of altering $MgCl_2$ and $MnCl_2$ concentration.

Relative activity (%)[1]				
	Mitochondrial enzyme		Cytoplasmic enzyme	
Ion concentration (mM)	$MgCl_2$	$MnCl_2$	$MgCl_2$	$MnCl_2$
0[2]	46	50	68	2
0.1	65	67	83	2
1	77	75	84	3
10	100	100	100	100
100	51	53	21	8

[1]For each cation relative activity for each enzyme is the ratio of incorporation of AMP achieved under the specific experimental conditions compared to the maximum incorporation of AMP achieved for that cation (expressed as a percentage).

[2]Each mitochondrial enzyme assay contained 0.08 mM $MgCl_2$ which was added as part of the enzyme fraction. Each cytoplasmic assay contained 0.8 mM $MgCl_2$ which was added as part of the enzyme fraction.

and cytosolic forms of the enzyme up to 0.2 mM CTP after which a decrease in incorporation of AMP is observed (Table 8).

TABLE 8. Effect of CTP concentration on nucleotidyl-transferase activity.

	Relative activity (%)[1]	
CTP concentration (mM)	Mitochondrial enzyme	Cytoplasmic enzyme
0	62	43
0.01	76	52
0.1	46	81
0.2	100	100
0.4	47	49
0.8	40	46
1	40	42
2	52	34

[1]Relative activity for each enzyme is the ratio of AMP incorporated at a specific CTP concentration compared to the maximum incorporation of AMP achieved under the specified conditions for that enzyme (expressed as a percentage).

DISCUSSION

This study was initiated to characterize the mitochondrial tRNA nucleotidyltransferase and explore the possibility that the mitochondrial and cytosolic tRNA nucleotidyltransferases in wheat were products of the same gene as has been shown to be the case in *Saccharomyces cerevisiae* (Chen *et al.*, 1992). To do this both the cytosolic and mitochondrial tRNA nucleotidyltransferases from wheat embryos were partially purified and their characteristics compared.

Although our lab had previously isolated and characterized the cytosolic tRNA nucleotidyltransferase from lupin (Shanmugam *et al.*, 1996) wheat embryos were chosen as the model plant system for this study because both a cytosolic (Dullin *et al*, 1975) and mitochondrial (Hanic-Joyce and Gray, 1990) tRNA nucleotidyltransferase had been purified or partially purified from this tissue. A primary reason for using wheat embryos is that previous studies have shown that mitochondria isolated from dark grown wheat embryos are significantly free of chloroplast contamination (Joyce and Gray, 1989). This was a major concern as we initially wished to characterize the mitochondrial form of the enzyme devoid of any chloroplast contamination. Although it is clear that chloroplasts must also have a tRNA nucleotidyltransferase to synthesize mature tRNAs, I wanted to compare initially the cytosolic and mitochondrial tRNA nucleotidyltransferases as the mitochondrial and cytosolic forms of this enzyme have been shown to be shared in yeast (Chen et al., 1992). Additionally, there is precedent for other tRNA maturation enzymes including isopentenyl pyrophosphate transferase (Gillman *et al*, 1991), and N^2,N^2-dimethylguanosine-specific tRNA methyltransferase (Li *et al.*, 1989) being shared between mitochondria and the nucleus in yeast. In yeast it is also clear that histidyl-tRNA synthetase (Natsoulis and Fink, 1986) and valyl-tRNA synthetase (Chatton et al, 1988) are shared between the cytosol and the mitochondria. Perhaps more significantly, it has

recently been shown that the mitochondrial and cytosolic alanyl-tRNA synthetases in *Arabidopsis* are products of a single gene (Mireau *et al.*, 1996).

These numerous examples of tRNA maturation enzymes (including tRNA methyltransferase) of yeast and plant that are shared between mitochondria and other cellular locations suggest the possibility that this might also be the case for tRNA nucleotidyltransferase in wheat. Additionally, the fact that tRNAs must be imported into plant mitochondria but not chloroplasts makes the study of the import of enzymes involved in tRNA maturation into mitochondria more interesting than the import of the same enzymes into chloroplasts as these enzymes may actually be involved in the import of tRNAs into mitochondria. Recently, (Tarassov *et al.*, 1995) it has been shown that the mitochondrial lysyl-tRNA synthetase is involved in import of a lysine-tRNA into yeast mitochondria. Here, mitochondrial tRNA nucleotidyltransferase was studied in wheat because this enzyme must interact with all tRNAs and therefore may be involved in tRNA import into mitochondria.

1. PURIFICATION OF tRNA NUCLEOTIDYLTRANSFERASE

A) Cell fractionation

Initially cell fractionation was performed on the wheat embryo extracts in order to isolate different cellular compartments. This first step in protein purification showed that the majority (80%) of the tRNA nucleotidyltransferase activity in the cell was found in the cytosolic fraction while relatively little activity was found in either the mitochondrial or nuclear fractions (5% and 15% respectively) (Table 1). This is consistent with what has been observed in *S. cerevisiae* where immunofluorescence microscopy indicated that the majority of the tRNA nucleotidyltransferase was cytosolic (Wolfe *et al.*, 1996). In yeast it appears that this enzyme contains a weak nuclear localization signal so that it is exported more efficiently from the nucleus than it is imported into the nucleus leading to the low levels of nuclear enzyme (Wolfe *et al.*, 1996). While the level of tRNA

nucleotidyltransferase is low in yeast nuclei (Wolfe et al., 1996) it is clear that this enzyme functions there as nuclear-restricted tRNA precursors have been isolated with intact CCA ends (Knapp *et al.*, 1978, Peebles *et al.*, 1979, Wolfe et al., 1996). It is, therefore, likely that tRNA nucleotidyltransferase plays a role in the nucleus in plants as well. Additionally, in *Xenopus* oocytes about 25% of the detectable tRNA nucleotidyltransferase activity was found in the nucleus (Solari and Deutscher, 1982) This observation is in good agreement with the 15% activity observed here (Table 1). This suggests a nuclear function for the enzyme. The level of nuclear tRNA nucleotidyltransferase observed in wheat may be a true indication of the natural distribution of this enzyme in plants. However, at this point I can not rule out the possibility that it also may reflect leakage of the tRNA nucleotidyltransferase from the nucleus during the cell fractionation procedure Similar observations of leakage from nuclear fractions were made for nuclei isolated from *Xenopus* oocytes and rat liver (Solari and Deutscher, 1982, Mukerji and Deutscher, 1972). Since the nuclear fractions that were used here were stored before they could be tested for enzyme activity it is probable that some of the nuclei ruptured causing the enzyme to leak out. In fact, it has been shown that the enzyme readily leaks out of purified *Xenopus* oocyte nuclei on storage (Solari and Deutscher, 1982). The overall low level of this enzyme in cells (Shanmugam *et al*., 1996) and specifically in nuclei (Wolfe *et al*., 1996) and the fact that this enzyme can easily leak out of stored nuclei probably explain the earlier observation that rat liver nuclei lack tRNA nucleotidyltransferase (Mukerji and Deutscher, 1972) and may reflect the low level of nuclear enzyme observed here

Some of the nuclear tRNA nucleotidyltransferase also may have been lost through sample preparation. Since the wheat nuclear extract was distributed among several centrifuge tubes recovery of all the nuclei may not have been achieved The enzyme also may not have been stable under these conditions.

Although yeast mitochondria have been shown to contain low levels of tRNA nucleotidyltransferase (Wolfe *et al.*, 1996) it is clear that this enzyme is essential for

mitochondrial tRNA maturation (Chen *et al.*, 1992). The relatively low level of mitochondrial tRNA nucleotidyltransferase activity seen here (Table 1) is consistent with the low level of tRNA nucleotidyltransferase in yeast mitochondria and may indicate that the natural distribution of this enzyme in plants is similar to that in yeast. Again it is possible that some of the mitochondria have lyzed during the fractionation procedure as approximately 33% of the detectable tRNA nucleotidyltransferase activity in rat liver was associated with mitochondria (Mukerji and Deutscher, 1972). The fact that 50% of the tRNA nucleotidyltransferase activity was associated with the mitochondria before lysis (Table 2) supports the idea that some mitochondria were lyzed during the purification procedure. An alternative explanation for the high levels of tRNA nucleotidyltransferase activity seen in the mitochondrial fraction before lysis may be contamination of this fraction with some other form of tRNA nucleotidyltransferase, possibly cytosolic or chloroplast. The former explanation seems more probable for the following reasons. First, we isolated our mitochondria from wheat embryos using a protocol that had been shown to prevent significant chloroplast contamination of mitochondria (Joyce and Gray, 1989) such that contamination of the mitochondrial enzyme extract with chloroplast tRNA nucleotidyltransferase seems unlikely. Second, the contamination of the mitochondrial enzyme with the cytosolic tRNA nucleotidyltransferase is also unlikely since the characteristics exhibited by the mitochondrial and cytosolic enzymes throughout the purification protocol are quite different (see results) and we would have observed differences in the characteristics of the enzyme in contaminated mitochondrial preparations. It is possible, however, that some mitochondria lyzed during their preparation such that some of the mitochondrial enzyme may have contaminated what was designated the cytosolic enzyme. With reference to this it is interesting that a small peak of enzyme activity elutes during hydroxylapatite chromatography of the cytosolic enzyme at approximately 80 mM sodium phosphate (Figure 7). This peak is in the same region as the major activity peak for the mitochondrial enzyme from the hydroxylapatite column (Figure

6). One possible explanation for the activity pattern of the cytosolic enzyme is that the cytosolic fraction was contaminated with the mitochondrial enzyme during its preparation.

Mitochondrial lysis (Table 2) resulted in both an increase in total protein (by about 40%) and in tRNA nucleotidyltransferase activity (by about 100%) as expected if proteins including the tRNA nucleotidyltransferase were contained within the mitochondrial membrane. Based on these observations it seems likely that the activity being detected in mitochondria prior to lysis (Table 2) is due to breakage of mitochondria during preparation and storage. The stress of freezing and thawing the mitochondria may have caused some lysis as well as some inactivation of enzyme. Preliminary experiments to determine how storage at -70^{0}C and freezing and thawing of enzyme extracts affect enzyme activity indicated a 5% decrease in enzyme activity after 4 hours at -70^{0}C (data not shown). It is possible that longer term storage of the extracts could have a proportional effect on enzyme activity, although enzyme extracts have shown high levels of activity after twelve months of storage at -70^{0}C.

The mitochondrial tRNA nucleotidyltransferase enzyme appears to be a soluble enzyme within the mitochondrion since more than 90% of the activity recovered from the mitochondria was found in the soluble fraction (Table 2) as had previously been shown (Hanic-Joyce and Gray, 1990). This is also in good agreement with results from potato (Marchfelder and Brennicke, 1994), rat liver (Mukerji and Deutscher, 1972) and yeast (Chen *et al.*, 1992) mitochondria indicating that tRNA nucleotidyltransferase is present in the mitochondrial matrix. Of the activity present in the lyzed mitochondrial fraction only 73% is recovered after centrifugation at 100 000 X g (67% in the S100 and 6% in the P100). One explanation for the loss of activity is that some of the enzyme was inactivated by handling or by degradation. Proteases may have been introduced during handling of the extracts or the enzyme may not have been stable under the conditions used. Mitochondrial fractionation did not yield any increase in specific activity (Table 2). This is perhaps not

surprising since although 90% of the protein was contained in the S100 fraction approximately one quarter of the total enzyme activity was lost at this step.

B) Ammonium sulfate fractionation

The next step used in our protein purification scheme was ammonium sulfate fractionation. This procedure makes use of the different solubilities of proteins in solutions of salt. Ammonium sulfate was used because it is the most widely used salt in fractionation due to its protein-stabilizing properties. This step not only served as an initial purification step to eliminate some contaminating proteins, but it also served to concentrate the target proteins in a solution of smaller volume, which is more suitable for column chromatography. The majority of tRNA nucleotidyltransferase activity for both the cytosolic and mitochondrial enzymes was found in the 20-40% (w/v) ammonium sulfate fractions (Table 3). This is in good agreement with what had been seen previously for the wheat mitochondrial enzyme in which the majority of the tRNA nucleotidyltransferase activity was seen in the 20-30% (w/v) and 30-50% (w/v) ammonium sulfate fractions (Hanic-Joyce and Gray, 1990). The results also agree well with the lupin cytosolic enzyme (Shanmugam *et al*, 1996) where the majority of the lupin tRNA nucleotidyltransferase activity was isolated between 30 and 55% ammonium sulfate saturation (16-31% w/v). This step resulted in a 3-fold enrichment of the mitochondrial enzyme and a 2-fold enrichment of the cytosolic enzyme. These results are typical of those observed for other tRNA nucleotidyltransferases including a 2.1-fold purification of the *E.coli* enzyme (Schofield and Williams, 1977), a 2.2-fold purification for the *Lupinus luteus* tRNA nucleotidyltransferase (Cudny *et al.*, 1978), and a 1.8-fold purification for the *Lupinus albus* enzyme (Shanmugam *et al.*, 1996).

Ammonium sulfate fractionation resulted in a 14% loss of the cytosolic tRNA nucleotidyltransferase activity and only a 1.3% loss of mitochondrial activity (Table 3). This step in the purification procedure resulted in an 11% loss of mitochondrial protein

and a 24% loss of the cytosolic protein. The unrecovered protein may have been lost during dialysis as it has been reported (Deutscher, 1972b) that tRNA nucleotidyltransferase binds to dialysis tubing. In any event these observations indicate a greater loss of the cytosolic enzyme and perhaps indicate that the cytosolic enzyme is less stable than its mitochondrial counterpart.

C) DEAE - Sepharose chromatography

Column chromatography was used next to fractionate the mixture of proteins obtained from ammonium sulfate fractionation. The first chromatographic step used was DEAE-Sepharose chromatography. DEAE-Sepharose resin consists of positively charged diethylaminoethyl groups covalently linked to Sepharose which binds negatively charged macromolecules (Scopes, 1987). DEAE-Sepharose resin was used in the purification of tRNA nucleotidyltransferase since it had been used successfully in the purification of other tRNA nucleotidyltransferases including those from lupin (Shanmugam *et al.*, 1996, Cudny *et al.*, 1978), and rabbit liver (Deutscher, 1972b). The mitochondrial and cytoplsmic enzymes showed similar characteristics on the DEAE-Sepharose column. Both enzymes eluted from the column at approximately 200 mM KCl (Figure 4 and Figure 5). Previously, wheat mitochondrial tRNA nucleotidyltransferase had been shown to elute from a DEAE-Sephacel column with 100 mM and 200 mM KCl fractions (Hanic-Joyce and Gray, 1990) while potato mitochondrial enzyme eluted from a DEAE-Trisacryl column with 200 mM KCl (Marchfelder and Brennicke, 1994) in good agreement with what was observed in this study. This purification step resulted in a 7.5-fold purification of the mitochondrial enzyme (Table 4), while only a two-fold purification was achieved for the cytosolic enzyme (Table 5). While the fold purification achieved for the mitochondrial tRNA nucleotidyltransferase is typical of that obtained by other tRNA nucleotidyltransferases (Shanmugam *et al.*, 1996, Cudny *et al.*, 1978) this purification step was less successful for the cytosolic enzyme. The main reason for this is that the activity

peak of the cytosolic tRNA nucleotidyltransferase coincided more closely with the protein peak than did the mitochondrial enzyme such that fewer contaminating proteins were removed (Figure 5). DEAE chromatography resulted in a 44% recovery of activity for the mitochondrial protein (Table 4) and a 22% recovery if activity for the cytosolic enzyme (Table 5). This again suggests that the cytosolic enzyme is less stable than the mitochondrial enzyme. The loss of activity again could have been due to protein binding to dialysis tubing during dialysis. Another possibility is that not all the enzyme was eluted from the column, although this is unlikely since the upper limit of the elution gradient contained 500 mM KCl. In any event, the level of cytosolic activity recovered during this purification step is comparable to that obtained with cytosolic tRNA nucleotidyltransferases from *Lupinus albus* (46%) (Shanmugam *et al.*, 1996), *Lupinus luteus* (46%) (Cudny *et al.*, 1978) and wheat embryos (55%) (Dullin *et al.*, 1975).

Since both the mitochondrial and cytosolic proteins bound to the DEAE resin and required 200 mM KCl to be eluted, this suggests that the proteins are negatively charged at pH 8.5. To this point in the purification scheme there were no apparent differences between the mitochondrial and cytosolic forms of this enzyme.

D) Hydroxylapatite chromatography

Hydroxylapatite chromatography was the second chromatographic step used in the purification scheme. While the method of protein binding and elution on hydroxylapatite has not yet been fully understood, this step was included in the purification scheme since it had been proven successful in the purification of tRNA nucleotidyltransferases from other sources including *Lupinus albus* (Shanmugam *et al.*, 1996), *Lupinus luteus* (Cudny *et al.*, 1978), rabbit liver (Deutscher, 1972b), yeast (Chen *et al.*, 1990) and *E.coli* (Schofield and Williams, 1977).

In contrast to what was observed with DEAE chromatography, the mitochondrial and cytosolic enzymes eluted from the hydroxylapatite columns at different ionic strengths

(compare Figure 6 and Figure 7). The mitochondrial enzyme eluted from the hydroxylapatite column between 75-150 mM sodium phosphate, while the cytosolic enzyme eluted between 150-240 mM sodium phosphate. This is the first evidence of two tRNA nucleotidyltransferases within one organism showing different characteristics on hydroxylapatite chromatography. The yeast cytosolic and mitochondrial tRNA nucleotidyltransferases showed similar characteristics on DEAE cellulose and hydroxylapatite chromatography (Chen *et al.*, 1990, Chen *et al*., 1992), but this is not surprising since they have been shown to be products of a single gene (Chen *et al.*, 1992). It is interesting that both tRNA nucleotidyltransferases (presumbly mitochondrial and cytosolic) from rabbit liver showed similar HA elution profiles (Deutscher, 1972) since although they had similar molecular weights they were shown to elute separately from phosphocellulose column chromatography (Deutscher, 1972a) and showed some differences in their catalytic properties (Deutscher, 1972b) indicating that they are different proteins.

As was pointed out earlier, a small peak of enzyme activity elutes during HA chromatography of the cytosolic enzyme at approximately 80 mM sodium phosphate (Figure 7). This peak is in the same region as the major activity peak for the mitochondrial enzyme from the hydroxylapatite column (Figure 6). As suggested earlier one possible explanation for this observation is that this second peak represents some mitochondrial contamination of the cytosolic fraction which could have occurred during cell fractionation. If some mitochondria lysed during its preparation, then perhaps some of the mitochondrial enzyme has contaminated what we call the cytosolic fraction.

Hydroxylapatite chromatography resulted in a 32-fold enrichment over the crude extract for the mitochondrial enzyme (Table 4), while only a 4-fold enrichment was seen for the cytosolic enzyme (Table 5). A possible explanation for the great difference between the fold purification of the two enzymes is that during the purification of the mitochondrial enzyme a 250 ml gradient was used to elute bound proteins, with fraction

sizes of 1.5 ml while a 250 ml gradient was used to collect bound proteins with 10 ml fractions collected during the purification of the cytosolic enzyme. Thus, better resolution was obtained for the mitochondrial enzyme than with the cytosolic enzyme. Coupled with the fact that the amount of protein retained on the HA column during the purification of the cytosolic enzyme was 65% of what was loaded while the amount of protein retained on the HA column during the purification of the mitochondrial enzyme was only 36% of what was loaded each active fraction collected during purification of the cytosolic enzyme contained many more proteins than its mitochondrial counterpart. Hydroxylapatite chromatography resulted in a 40% loss of mitochondrial enzyme activity and a 74% loss of cytosolic enzyme activity. These results are comparable with activity loss observed with hydroxylapatite chromatography during the purification of tRNA nucleotidyltransferases from other sources such as a 48% loss for the *E.coli* enzyme (Schofield and Williams, 1977), a 74% loss for the *Lupinus luteus* enzyme (Cudny *et al.*, 1978), and a 47% loss of enzyme activity for the yeast enzyme (Chen *et al.*, 1992). In contrast, tRNA nucleotidyltransferase from *Lupinus albus* showed a 65% increase in enzyme activity with this purification step (Shanmugam *et al.*, 1996). One difference between the protocol used in the purification of the *Lupinus albus* protein and that used in this work is the salt used in protein elution. The former employed potassium phosphate, whereas in the latter sodium phosphate was used. Sodium phosphate was used in this study since it was the salt recommended by the makers of the hydroxylapatite (Pharmacia). This substitution may account for the different recovery of enzyme activity observed between the two protocols if tRNA nucleotidyltransferase is more stable in potassium phosphate. It is possible that tRNA nucleotidyltransferase is more susceptible to inactivation in the presence of 200 mM sodium phosphate than in a lower concentration of potassium phosphate since the lupin enzyme eluted from the HA column at approximately 100 mM potassium phosphate. In fact, when enzyme activity was measured in the presence of different concentrations of

NaCl or KCl, enzyme activity was significantly lower in the presence of 200 mM salt as compared to 100 mM salt (Table 6).

The greater loss of activity observed for the cytosolic enzyme may be due to the fact that it eluted from the column at the end of the elution gradient, so that the possibility exists that some of the cytosolic tRNA nucleotidyltransferase remained on the column. Although an effort was made to elute any protein remaining on the HA column, the low solubility of sodium phosphate at concentrations above 250 mM at 4°C made this very difficult.

E) tRNA affinity chromatography

The final chromatographic step in the purification of tRNA nucleotidyltransferase was tRNA affinity chromatography. Both the mitochondrial and cytosolic enzymes showed similar elution profiles from the affinity column. Both proteins were eluted from the column after sodium chloride and EDTA had been added to the elution buffer (Figure 8 and Figure 11). This is in good agreement with what had been observed for the *Lupinus albus* enzyme (Shanmugam *et al.*, 1996), and the wheat cytosolic enzyme which was eluted from a tRNA-hydrazyl-Sepharose 4B column with 200 mM KCl (Dullin *et al.*, 1975). Since the tRNA-affinity column specifically binds tRNA-binding proteins it is not surprising that the majority of proteins were not bound to the tRNA affinity column However, only 27.5% of the mitochondrial enzyme activity and 5% of the cytosolic enzyme activity were recovered in this step The most probable reason for this observation is that some enzyme was retained on the column. Since the coupling of the tRNA molecules onto the affinity resin could not be controlled, the orientation of the bound tRNAs was not uniform. As such, some of the tRNA nucleotidyltransferase may have been more tightly bound to the affinity column such that it was not eluted in the presence of 200 mM NaCl. These results are comparable with the *Lupinus albus* enzyme (Shanmugam *et al.*, 1996) which showed a 5% enzyme recovery and with the yeast

mitochondrial enzyme (Chen *et al.*, 1990) which showed a 33% recovery in activity with this purification step. However, there are a few important differences between the two protocols used that may account for the variability in enzyme recovery. Primarily, in the purification of the yeast enzyme, the affinity column was generated with tRNAs with uniform 3'-ends such that the CCA sequence was removed. This results in an increase in affinity of the tRNA nucleotidyltransferase to the bound tRNAs to the gel.

Affinity chromatography resulted in greater than 370-fold enrichment in enzyme activity over the crude extract for the mitochondrial enzyme, with only a 113-fold enrichment achieved for the cytosolic enzyme (Table 5). These data are not consistent with enrichment values obtained with cytosolic tRNA nucleotidyltransferases from other sources including an 11 800-fold enrichment obtained with the *E.coli* enzyme (Schofield and Williams, 1977), a 2 000-fold enrichment with the *Lupinus luteus* enzyme (Cudny *et al*, 1978), and a 10 000-fold enrichment with the *Lupinus albus* enzyme (Shanmugam *et al.*, 1996) but are in good agreement with what was seen for the yeast mitochondrial enzyme (166-fold enrichment, Chen *et al.*, 1992). The extremely poor recovery of the wheat cytosolic enzyme may reflect the stability of this enzyme.

F) Stability of tRNA nucleotidyltransferase

i) cytosolic protein

A previous purification scheme for the wheat cytosolic enzyme has shown a 7000-fold increase in specific activity (Dullin *et al.*, 1975) while only a 113-fold increase (Table 5) was achieved in this study. Thus, the purification procedure described here is clearly not the most efficient to isolate the cytosolic form of this enzyme from wheat. This protocol was used, however, because we wanted a direct comparison between the mitochondrial and cytosolic enzyme within one organism. This protocol also had proven effective in isolating the cytosolic tRNA nucleotidyltransferase from lupin, in which a 10 000-fold purification was achieved over crude extracts (Shanmugam *et al.*, 1996). A

comparison of the purification of the lupin enzyme to that of the wheat enzyme indicates that the increase in specific activity is less at every step of the purification of the wheat enzyme as compared to the lupin enzyme. The wheat enzyme eluted from the DEAE and HA columns at higher ion concentrations that the lupin enzyme (the wheat enzyme eluted at 205 mM KCl off the DEAE column [Figure 4], while the lupin enzyme eluted at 60 mM KCl [Shanmugam *et al.*, 1996]). Since we have determined that the wheat cytosolic enzyme activity is inhibited by 60% in the presence of 200 mM KCl (Table 6), the higher salt concentration in these fractions may contribute to the low level of activity seen such that the specific activity obtained with DEAE chromatography is not a true indication of the enzyme present. Also, while the lupin enzyme eluted from the HA and tRNA affinity columns at the tail of the protein profile, the wheat enzyme eluted in earlier fractions from both these columns (Figure 7 and Figure 11). This observation may be due to the different salt buffers used during protein elution. While we used sodium phosphate to elute the wheat enzyme, potassium phosphate was used to elute absorbed proteins from the hydroxylapatite column in the purification of the lupin enzyme. The wheat enzyme appears to be less stable through these purification protocols than does the lupin enzyme As indicated previously, approximately 15% of the wheat cytosolic tRNA nucleotidyltransferase activity was lost during ammonium sulfate fractionation (Table 2). More than 75% of the tRNA nucleotidyltransferase activity remaining was lost in DEAE and HA chromatography such that only 5% of the enzyme activity remained after hydroxylapatite chromatography This compares with almost 70% of enzyme activity remaining with the lupin enzyme after HA chromatography (Shanmugam *et al.*, 1996). However, with both the wheat and lupin cytosolic tRNA nucleotidyltransferases more than 95% of enzyme activity was lost after tRNA affinity chromatography, indicating that only a small amount of activity remains after this purification protocol.

Possible reasons for the overall loss of enzyme activity seen may be that an important co-factor is being dialyzed out, or that dialysis at $4^{0}C$ causes some enzyme

inactivation. Dialysis was used to change the buffer of active fractions after each chromatographic step. In fact, preliminary studies showed that dialysis of active tRNA nucleotidyltransferase was decreased by 50% when dialyzed at 4°C for 8 hours (data not shown). A previous report (Dullin *et al.*, 1975) has shown that the wheat cytosolic tRNA nucleotidyltransferase is unstable and exhibits a 50% drop in activity when stored at -12°C for two weeks. Taken together these may suggest that the wheat cytosolic enzyme is unstable or is inactivated during our purification procedure.

ii) mitochondrial protein

The wheat mitochondrial enzyme was purified at least 370-fold with 5% of enzyme activity recovered (Table 2). This compares favorably with the yeast mitochondrial enzyme which was purified at least 166-fold with 7% recovery (Chen *et al.*, 1990). The increased yield and recovery observed with the wheat mitochondrial enzyme as compared to the cytosolic enzyme suggests either that the mitochondrial enzyme is more stable than the cytosolic enzyme, or that the mitochondrial extract lacks proteases or inhibitory substances that are present in the cytosolic extract. The increased loss of cytosolic enzyme activity as compared to mitochondrial enzyme activity may be a result of the increased time that the cytosolic enzyme must be kept at 4°C during the purification procedures. If these enzymes are subject to degradation at 4°C then the larger volumes used with the cytosolic fractions increase the time required to load and run the columns during purification of the cytosolic enzyme such that this enzyme spends more time at 4°C as compared to the mitochondrial enzyme. Also, since the volumes of the active cytosolic fractions were much larger at every purification step than their mitochondrial counterparts, longer dialysis times were required for the cytosolic enzyme. As mentioned previously, dialysis does greatly affect enzyme activity.

G) Molecular mass of tRNA nucleotidyltransferase

SDS-polyacrylamide gel electrophoresis was used to monitor the progress in the purification of tRNA nucleotidyltransferase. The number of different proteins in the active mitochondrial fractions decreased greatly over the course of the purification scheme (Figure 10). The fraction showing highest activity (Figure 10, lane 7) obtained from the final purification step showed two major protein bands at approximately 60 KDa. In contrast, the most active cytosolic fraction from the tRNA affinity column contained major protein bands at approximately 66 KDa (Figure 12, lane 4) which are absent from the active mitochondrial fraction (Figure 12, lane 5). Similar protein bands are present in fractions which show low or no enzyme activity (Figure 12, lanes 2 and 4) and, therefore, suggest that they are not the tRNA nucleotidyltransferase although we can not exclude the possibility that tRNA nucleotidyltransferase is present in this fraction but concealed by the other bands. The tRNA nucleotidyltransferase present in this fraction (Figure 12, lane 4) may also be below our level of detection. It is perhaps significant that the 60 KDa protein bands present in the active mitochondrial fraction (Figure 12, lane 5) are absent from the active cytosolic fraction (Figure 12, lane 4). Gel filtration data indicated a molecular mass of 66 KDa (Figure 11) for the cytosolic enzyme such that the enzyme in these fractions may be concealed by the other proteins of this molecular mass. A molecular mass of 60 KDa for the mitochondrial enzyme (Figure 11) is in good agreement with our observation of a major doublet around 60 KDa molecular mass on SDS-polyacrylamide gel electrophoresis. These results are in good agreement with the sizes predicted for tRNA nucleotidyltransferases from other sources. The yeast tRNA nucleotidyltransferase has an apparent molecular mass of 59 KDa on SDS-PAGE (Chen *et al.*, 1996), the rabbit liver tRNA nucleotidyltransferase had an apparent molecular mass of 70 KDa on SDS-PAGE (Deutscher, 1972a), while the *E.coli* enzyme was determined to be 52 KDa on SDS-PAGE (Schofield and Williams, 1977) and that of *Sulfolobus shibatae* was determined to be 48 KDa (Yue *et al.*, 1996). The apparent molecular mass of the tRNA nucleotidyltransferase from *Lupinus albus* was 66 KDa based on SDS-PAGE (Shanmugam *et al* , 1996) in good

agreement with the molecular mass predicted for the wheat cytosolic enzyme. These data support our hypothesis that we have carried out a purification of the mitochondrial tRNA nucleotidyltransferase such that it is a protein of approximately 60 KDa. In contrast, while the cytosolic enzyme has an apparent molecular mass of 66 KDa, our purification protocol does not allow us to detect this enzyme in a background of contaminating proteins. In any event, both the chromatographic (particularly hydroxylapatite) characteristics and apparent molecular masses of these two proteins suggest that they are different.

2. CHARACTERIZATION OF WHEAT tRNA NUCLEOTIDYL-TRANSFERASES.

In this study total wheat tRNA was used as a substrate for tRNA nucleotidyltransferase in all experiments. Since these tRNAs were not treated to remove the 3' terminal CCA that may have been added by the endogenous enzyme, a mixed population of tRNAs (which may contain N-, N-C, N-C-C or N-C-C-A 3' termini) exists in the reaction mixture Recent experiments with commercial wheat tRNAs suggest that 96% of the tRNA molecules in this preparation actually have an intact CCA end (Yue *et al.*, 1996). Although some uncertainty as to the exact number of intact CCA ends that are present makes precise kinetic parameters regarding the incorporation of radiolabeled AMP difficult, this uncertainty should not effect the general conclusions drawn since the same general population of wheat tRNAs was used in each experiment.

A) Time course

The effect of incubation time on the activity of both the mitochondrial and cytosolic enzymes was tested initially in order to determine the optimum reaction incubation time (data not shown). For both enzymes, a linear increase in the incorporation of AMP was observed until the 30 minute mark, after which ATP incorporation reached a

plateau. This suggests that by 30 minutes, the enzyme had used up most of the substrate An incubation time of 25 minutes was subsequently chosen for all further experiments. This type of time span was also used for the *E.coli* tRNA nucleotidyltransferase (Williams and Schofield, 1977), and is similar to the 20 minutes used for the lupin tRNA nucleotidyltransferase (Shanmugam *et al.*, 1996).

B) Effect of radiolabeled ATP concentration on enzyme activity

Initially, tRNA nucleotidyltransferase activity was measured using the protocol of Shanmugam *et al.* (1996), but the level of AMP incorporation was not appreciably above background. This suggests that the wheat enzyme was less active than its lupin homologue. This may be due to the wheat enzyme being less stable than its lupin counterpart as indicated in the recovery during the purification procedure (Table 4 and 5) To correct this, experiments were performed with increasing amounts of radiolabeled ATP and/or decreasing amounts of non-radiolabeled ATP. It was shown that optimum incorporation was seen when the amount of non-radiolabeled ATP was decreased by a factor of 10 (from 0.2 mM to 0.02 mM) and the amount of radiolabeled ATP was increased 10-fold (from 0.015 µM to 0.15 µM [α^{32}P] ATP) (data not shown)

C) Effect of CTP concentration

In the presence of 0.15 µM [α^{32}P] ATP, maximum incorporation of radioactive ATP was shown in the presence of 0.2 mM CTP for both the mitochondrial and cytosolic tRNA nucleotidyltransferases. Concentrations above 0.2 mM CTP resulted in a decrease in the incorporation of AMP (Table 8). One explanation for this observation is that at levels of CTP up to and including 0.2 mM, the CTP is acting as substrate while at higher concentrations it competes with ATP for addition at the third position, resulting in CCC instead of CCA addition at the 3' end of tRNA Similar results have been seen with other tRNA nucleotidyltransferase (Deutscher, 1972b, Shanmugam, 1994).

D) Effect of pH on enzyme activity

Maximal activity was seen between pH 8.5 and 10 in glycine buffer (Figure 13) for both the cytosolic and mitochondrial tRNA nucleotidyltransferases, although the pH optimum of the cytosolic enzyme seems to be at pH 9.5, while that of the mitochondrial enzyme occurred at pH 9. In order to verify whether this minor difference in pH optimum was real, the experiment was repeated several times, with both enzymes tested concurrently. In fact, this difference in pH optima was observed every time, indicating that the two enzymes have slightly different pH optimum in glycine buffer under the conditions used here. This observation, however, may be due to the increased amount of Tris-HCl (pH 8.5) that is added with the cytosolic enzyme compared to that added with the mitochondrial enzyme. Only 1µl of mitochondrial extract in 50 mM Tris-HCl (pH 8.5) was added to each 100 µl mitochondrial assay mixture while 10 µl of cytosolic extract containing 50 mM Tris-HCl (pH 8.5) was added to each 100 µl cytosolic assay mixture. This results in a final concentration of 0.5 mM Tris-HCl (pH 8.5) in the reaction mixture for the mitochondrial enzyme compared to a final concentration of 5 mM Tris-HCl (pH 8.5) for the cytosolic assays in addition to the 100 mM glycine buffer added at each pH. This Tris-HCl (pH 8.5) may alter the actual pH of the reaction mixture such that glycine buffer at a more alkaline pH is needed to give an accurate reaction at pH 9. In any event a pH optimum of approximately 9 in glycine buffer observed for the wheat tRNA nucleotidyltransferases is comparable with what has been seen in other organisms, such as *E.coli* (Carre *et al.*, 1970), yeast (Chen *et al.*, 1990), *Musca domestica* (Poblete *et al.*, 1977), rabbit liver (Deutscher, 1972b, Maisiakowski and Deutscher, 1980), *Lupinus luteus* (Cudny *et al.*, 1978b) and *Lupinus albus* (Shanmugam *et al.*, 1996).

The pH optima of the mitochondrial and cytosolic tRNA nucleotidyltransferases also were tested in Tris buffer. A biphasic activity profile was seen for both enzymes with the first pH optimum of the cytosolic enzyme approximately 0.5 pH units greater than that

of the mitochondrial enzyme (Figure 14). Although this result was repeatable the slight differences in the pH optimum between the mitochondrial and cytosolic tRNA nucleotidyltransferase in Tris buffer may again be due to the fact that 10X more of the cytosolic fraction containing Tris-HCl (pH 8.5) was assayed than the mitochondrial fraction. A biphasic activity profile seen with Tris-HCl buffer is comparable with what had been seen previously for the wheat cytosolic enzyme (Dullin *et al.*, 1975) which showed a maximum activity at pH 7.6 and half-maximal activity at pH 8.6 as is seen here.

While it is possible that there is a minor difference in pH optimum for the mitochondrial and cytosolic enzymes from wheat it would not be surprising if no difference was seen. Transfer RNA nucleotidyltransferases from eubacteria (Carre *et al.*, 1970), eukaryotes (Cudny *et al.*, 1978a, Masiakowski and Deutscher, 1980, Shanmugam *et al.*, 1996), archaebacteria (Yue *et al.*, 1996) and organelles (Chen *et al.*, 1990) all show remarkably similar pH optima (approximately from pH 9-9.5) indicating that this feature of these enzymes has been conserved over time. Therefore, pH optimum is probably not the best criterion to use to define differences or similarities between these enzymes. A marked difference in pH optimum would have been significant, but similar pH optimum for the two enzymes is not particularly informative.

c) Requirements for metal ions

Both KCl or NaCl at concentrations greater than 100 mM showed an inhibitory effect on the activity of tRNA nucleotidyltransferase (Table 6) This result had also been observed for tRNA nucleotidyltransferases from *Lupinus luteus* (Cudny *et al.*, 1978b), *E.coli* (Carre *et al.*, 1970) and rabbit liver (Deutscher, 1972) where salt concentrations above 150 mM were inhibitory and for the wheat mitochondrial enzyme where KCl concentrations above 200 mM are inhibitory (Hanic-Joyce and Gray, 1990). While activity drops more quickly with increasing NaCl concentrations for the mitochondrial enzyme (41% drop in activity by 100 mM), the cytosolic enzyme shows a significantly larger level

of inhibition at 400 mM NaCl (92%) than does the mitochondrial enzyme (64% drop). The same trend is seen in the presence of KCl, such that lower concentrations of salt have a greater inhibitory effect on the mitochondrial enzyme, while larger concentrations of salt have a greater inhibitory effect on the cytosolic enzyme. Therefore, while both the mitochondrial and cytosolic tRNA nucleotidyltransferases are inhibited by concentrations of NaCl or KCl at or above 100 mM, the inhibitory effects became greater on the cytosolic enzyme than on the mitochondrial enzyme with increasing concentrations of salt. Again, these observations suggest differences between the mitochondrial enzyme and its cytosolic counterpart.

Maximal activity has been shown for many tRNA nucleotidyltransferases at approximately 10 mM $MgCl_2$ concentration (Carre *et al.*, 1970, Deutscher, 1972b, Dullin *et al.*, 1975, Cudny *et al.*, 1978b, Shanmugam *et al.*, 1996) as was observed for the wheat enzymes (Table 7). Although $MnCl_2$ has been shown to replace $MgCl_2$ with tRNA nucleotidyltransferases from other sources, maximal activity usually has been achieved with approximately 0.1 mM (Carre *et al.*, 1970) to 1 mM (Dullin *et al.*, 1975) $MnCl_2$ in contrast to the 10 mM $MnCl_2$ which showed maximal activity under the experimental conditions used here (Table 7). The effect of $MnCl_2$ on cytosolic tRNA nucleotidyltransferase activity is even more striking under our assay conditions in that an increase of 97% in activity is seen in the presence of 10 mM $MnCl_2$ as compared to 1 mM $MnCl_2$ (Table 7). Previously the wheat cytosolic enzyme had been shown to have maximal activity at approximately 1 mM M (Dullin *et al.*, 1975) with this level of activity approximately equal to the maximal activity seen with 10 mM $MgCl_2$. The differences between these results and what was observed in our study may be due to the differences in the assay systems used. While Dullin *et al.* (1975) pretreated their tRNA such that they could measure incorporation of radiolabeled CTP, here ATP incorporation was measured using tRNA which had not been treated with exonuclease and thus contained X-C, X-CC and X-CCA termini. Therefore, our activity assay essentially

measures AMP incorporation with no actual input from the addition of C- or C-C sequences at the 3'-end of tRNAs since as previously mentioned most (96%) of our tRNA substrate already contain the C-C sequence. It is possible that manganese affects adenosine addition to a greater extent than it affects cytidine addition. It follows, therefore, that our assay would manifest these effects to a greater degree than the protocol used by Dullin *et al.* (1975). Also, while our assay contained 10 mM $MnCl_2$ it also contained 0.8 mM $MgCl_2$ which was added as part of the enzyme extract. An additive effect of $MgCl_2$ and $MnCl_2$ on enzyme activity has been seen previously with the *E.coli* enzyme (Carre *et al*, 1970) although it was not as dramatic as observed in this study.

3. PROTEIN IMPORT

Proteins which are shared between multiple intracellular locations are often the result of different transcription initiation sites being used in a gene which contains multiple in frame start codons (Wolfe *et al.*, 1994). The tRNA nucleotidyltransferase gene in yeast contains three in-frame start codons (Aebi *et al.*, 1990). Proteins synthesized from transcripts starting upstream of the first start codon are required for mitochondrial protein synthesis while shorter proteins arising from proteins synthesized from transcripts starting between the first and second codons provide the nucleocytosolic enzyme (Wolfe *et al.*, 1994). The additional amino acids resulting from translation of the longer transcripts provide information to target the protein to the mitochondrion. Previously (Shanmugam *et al.*, 1996), a lupin cDNA that encodes a cytosolic tRNA nucleotidyltransferase of 66 KDa molecular mass has been characterized. The size of the lupin cytosolic tRNA nucleotidyltransferase is in good agreement with the results presented in this report for the size of the wheat cytosolic enzyme. As in yeast this lupin cDNA contains a second in frame start codon (Shanmugam *et al.*, 1996) which if used in protein synthesis could result in the production of a second protein containing 57 fewer amino acids corresponding to a

decrease in molecular mass of approximately 6 KDa. Since this is the difference in size that is observed between the two wheat enzymes purified in this study, it is possible that two proteins could result from the lupin cDNA: one representing the mitochondrial enzyme containing these additional 57 amino acids which function as a mitochondrial targeting signal and a second representing the nucleocytoplasmic enzyme lacking the 57 amino acids. While the two forms of tRNA nucleotidyltransferase that we isolated from wheat had apparent molecular masses of 66 KDa and 60 KDa we found that the longer form was the cytosolic enzyme and the shorter form was the mitochondrial enzyme, the reverse of what would be predicted in lupin.

The simplest explanation of the differences in molecular mass between the cytosolic and mitochondrial enzymes that we observed is that these two proteins represent products of different genes The differences in the purification profiles and enzyme responses to salt between the two enzymes also support the hypothesis that these represent different proteins. The possibility remains, however, that these two proteins are products of the same gene and that the difference in size between the cytosolic and mitochondrial forms results from translation beginning at different start codons as described previously. The differences in the elution profiles of the two proteins from the HA column and responses to salt would then be dictated by the presence or absence of these extra amino acids. That these two proteins are products of different genes, however, seems more likely for the following reasons If the two proteins are products of the same gene, then the larger (and cytosolic) form of the enzyme is synthesized containing a mitochondrial targeting signal which directs some but not all of this protein to the mitochondrion where it is processed. It must then be explained how this enzyme is shared between the nucleocytoplasm and the mitochondrion. The yeast FUM1 gene product, fumarase, also has been shown to be shared between the cytosol and mitochondrion (Wu and Tzagoloff, 1987), however, in this case the cytosolic and mitochondrial forms of the enzyme have the same apparent molecular mass as all of the fumarase synthesized contains a mitochondrial targeting signal

and is targeted to the mitochondrion import machinery where it is processed before being distributed between the cytosol and mitochondrion (Stein *et al.*, 1994). Similarly, the yeast cytosolic and mitochondrial tRNA nucleotidyltransferases which are the products of the yeast CCA1 gene also have the same apparent molecular mass (Chen *et al.*, 1992) and it appears that while the mitochondrial form of the protein contains a mitochondrial targeting signal it is cleaved upon import to give a mature protein that is indistinguishable from the cytosolic form of the enzyme. In general, mitochondrial proteins are synthesized as larger precursors containing targeting signals which may be cleaved upon import (Glick and Schatz, 1991) while cytosolic proteins are synthesized as smaller proteins lacking this targeting signal (Bjork, 1995). My data is not consistent with this model. I have shown that the wheat cytosolic enzyme is larger than the wheat mitochondrial enzyme. It has recently been found that in *Arabidopsis* the same gene encodes both the cytosolic and mitochondrial alanyl-tRNA synthetases (Mireau *et al.*, 1996). The molecular mass predicated from the amino acid sequence for the cytosolic enzyme is 105 KDa, whereas that predicted for the presumed mitochondrial precursor is 110 KDa, however, removal of the presequence by the mitochondrial processing peptidase gives a polypetide similar in size to the cytosolic enzyme. Again, this is not the case with the wheat tRNA nucleotidyltransferases purified in this study since the cytosolic enzyme is larger than the mitochondrial enzyme.

I can not as yet exclude the possibility that the import mechanism for the wheat mitochondrial tRNA nucleotidyltransferase is similar to that of the yeast *TRM1* gene product. *TRM1* as mentioned previously encodes an enzyme which catalyzes the modification of tRNAs at position 26 and functions in the cytosol, mitochondrion and nucleus (Martin and Hopper, 1994). TRM1p contains two in frame AUG start codons. Initiation at either start site results in enzymes which can be transported to the mitochondrion, however, while the mitochondrial targeting signal in the N terminal part of the shorter form of the enzyme is sufficient for mitochondrial localization, a 16-amino-

acid-long N-terminal extension present in the larger form enhances this signal and forms an enzyme which is more efficiently transported into the mitochondrion (Ellis *et al.*, 1987). As yet there is no evidence that this targeting signal is removed after import. It is possible as is the case for the *TRM1* gene products that wheat contains a single gene encoding the mitochondrial and cytosolic forms of tRNA nucleotidyltransferase and that the 66 KDa species represents a form of this protein that contains an inefficient mitochondrial targeting signal such that it is distributed between the mitochondria and the cytosol. When this enzyme enters the mitochondrion it has its mitochondrial sequence cleaved such that the 60 KDa species results. Further experiments must be carried out to resolve this issue.

4. CONCLUSIONS AND FUTURE WORK

In this study, the purification and characterization of the mitochondrial tRNA nucleotidyltransferase from *Triticum aestivum* is reported. As well, partial purification and characterization of the cytosolic form of this enzyme was carried out for comparative purposes. This is the first report in which two forms of this enzyme have been directly compared in plants. Although both the mitochondrial and cytosolic forms of the enzyme showed enzymatic characteristics typical of tRNA nucleotidyltransferases isolated from other sources it seems likely that they are products of different genes. This is based on the responses of these enzymes to NaCl, KCl and their responses to manganese ions Additionally, the cytosolic and mitochondrial enzymes also showed different behaviors on hydroxylapatite columns such that the mitochondrial enzyme eluted at 100 mM sodium phosphate while the cytosolic enzyme eluted at 200 mM sodium phosphate. Significantly, these two enzymes also differed in their apparent molecular masses. Gel filtration chromatography indicated a protein of 66 000 Da with maximal activity in the cytosolic fraction while a protein of 60 000 Da showed maximum activity in mitochondrial fractions. These results were also supported by SDS-PAGE analysis which showed differences in molecular mass of the major proteins in the active mitochondrial and cytosolic fractions.

While none of these data are alone conclusive, taken together they support the hypothesis that these enzymes are different proteins in wheat and suggest that unlike in yeast the mitochondrial and cytosolic forms of tRNA nucleotidyltransferase represent the products of different genes. This conclusion is also supported by previous data from the lupin tRNA nucleotidyltransferase which indicated that while the lupin tRNA nucleotidyltransferase could complement a nuclear defect in a yeast strain containing a temperature-sensitive tRNA nucleotidyltransferase, it was unable to support growth on a non-fermentable carbon source (Shanmugam *et al.*, 1996). One possible explanatior of this observation is that the lupin tRNA nucleotidyltransferase that was characterized is not imported into yeast mitochondria and is not the mitochondrial enzyme from lupin.

To show with a high degree of confidence that the wheat mitochondrial and cytosolic tRNA nucleotidyltransferases are products of different genes, cDNAs encoding the wheat cytosolic and mitochondrial tRNA nucleotidyltransferase must be cloned and sequenced and directly compared. As a first step towards this, peptides from proteins in the most active fractions of the mitochondrial extract from the tRNA affinity column are being sequenced. Preliminary sequence data suggest that one of the major bands may represent wheat mitochondrial histidyl-tRNA synthetase. Further protein sequence will be required to identify the other major protein.

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