Escherichia coli Dimethylallyl Diphosphate:tRNA Dimethylallyltransferase: A Binding Mechanism for Recombinant Enzyme[†]

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ABSTRACT: *Escherichia coli* dimethylallyl diphosphate:tRNA dimethylallyltransferase (DMAPP-tRNA transferase) catalyzes the first step in the biosynthesis of the hypermodified A37 residue in tRNAs that read codons beginning with uridine. The enzyme, encoded by the *miaA* gene, was overproduced and purified to apparent homogeneity in three steps by ion-exchange (DE52 and Mono-Q) and size exclusion chromatography. Affinity-tagged DMAPP-tRNA transferase containing a C-terminal tripeptide α-tubulin epitope also was overproduced and purified to apparent homogeneity in two steps by ion-exchange and immunoaffinity chromatography. Addition of the C-terminal tripeptide α-tubulin epitope to DMAPP-tRNA transferase did not affect the activity of the enzyme. Undermodified $tRNA^{Phe}$ used as substrate in the DMAPP-tRNA transferase-catalyzed reaction was isolated and purified from an overexpressing clone in a *miaA* deficient strain of *E. coli*. Active recombinant *E. coli* DMAPP-tRNA transferase is monomeric. The enzyme transferred the dimethylallyl moiety of DMAPP to A37, located adjacent to the anticodon in undermodified $tRNA^{Phe}$. The enzyme required Mg^{2+} for activity and exhibited a broad pH optimum. Michaelis constants for $tRNA^{Phe}$ and DMAPP are 96 ± 11 nM and 3.2 ± 0.5 μM, respectively, and V_{max} = 0.83 ± 0.02 μmol min^{-1} mg^{-1}. DMAPP-tRNA transferase bound $tRNA^{Phe}$ with a dissociation constant of 5.2 ± 1.2 nM. In contrast, DMAPP did not bind to the enzyme in the absence of tRNA. However, DMAPP was bound with a dissociation constant of 3.4 ± 0.6 μM in the presence of a minihelix analogue of the anticodon stem−loop of $tRNA^{Phe}$ where the base corresponding to A37 was replaced by inosine. These results suggest an ordered sequential mechanism for substrate binding.

Posttranscriptional modifications of nucleotides in tRNA confer unique properties to the macromolecule important for its biological function. Alkylation of the amino moiety of adenosine-37 (A37) by a dimethylallyl moiety is a ubiquitous modification in the tRNAs of prokaryotes and eukaryotes that read codons beginning with uridine. The enzyme responsible for this modification is dimethylallyl diphosphate:tRNA dimethylallyltransferase (DMAPP-tRNA transferase).[1]

Early literature referred to the hypermodification of A37 as the Δ^2 isopentenylation[2] of the tRNA. Thus, in yeast and mammalian systems, the modified residue was identified as N^6-(Δ^2-isopentenyl)adenosine (i^6A) (Kersten, 1984). In *Escherichia coli*, the primary derivative is 2-(methylthio)-N^6-(Δ^2-isopentenyl)adenosine (ms^2i^6A) (Kersten, 1984). Several other enterobacteriacae (Buck et al., 1982; Janzer et al., 1982) and plant-associated bacteria (Cherayil & Lipsett, 1977; Thimmappaya & Cherayil, 1974) contain the *cis* isomer of 2-(methylthio)-N^6-(4-hydroxy-Δ^2-isopentenyl)-adenosine (*cis*-ms^2io^6A) as the major component of the fully modified tRNA. In all cases, the first step in the biosynthesis of these hypermodified residues is the transfer of a dimethylallyl moiety, by DMAPP-tRNA transferase, to A37 in the full length polynucleotide.

The occurrence of i^6A or its derivatives in tRNA is not absolutely required for viability of the host organism. Bacterial and yeast strains lacking DMAPP-tRNA transferase are viable under standard growth conditions, although they exhibit altered cellular growth and translation rates (Eisenberg et al., 1979; Laten et al., 1978). Gefter and Russell (1969) originally demonstrated the need for these modified bases for the efficient interaction of tRNA with ribosomes. Subsequent studies indicated such modifications stabilize relatively weak A·U base pairings, thereby preventing misreading of the genetic code (Wilson & Roe, 1989). More recent studies point to roles far more subtle for modifications in tRNA. The level of modification at A37 has been implicated in the regulation of operon expression (Blum, 1988; Buck & Griffiths, 1982), aromatic amino acid uptake (Buck & Griffiths, 1981), and aerobiosis (Buck & Ames, 1984) and recently in the regulation of *vir* gene expression in *Agrobacterium tumefaciens* (Gray et al., 1992). There is also increasing evidence that the extent of modification at

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[1] Abbreviations: Amp, ampicillin; BD-cellulose, benzoylated DEAE-cellulose; BME, 2-mercaptoethanol; BSA, bovine serum albumin; *cis*-ms^2io^6A, *cis*-2-(methylthio)-N^6-(4-hydroxy-Δ^2-isopentenyl)adenosine; DEPC, diethyl pyrocarbonate; DMAPP, dimethylallyl diphosphate; DMAPP-tRNA transferase, dimethylallyl diphosphate: tRNA dimethylallyltransferase; EDTA, ethylenediaminetetraacetic acid; i^6A, N^6-(Δ^2-isopentenyl)adenosine; IPP, isopentenyl diphosphate; IPP isomerase, isopentenyl diphosphate:dimethylallyl diphosphate isomerase; IPTG, isopropyl thio-β-D-galactoside; ms^2i^6A, 2-(methylthio)-N^6-(Δ^2-isopentenyl)adenosine; NACS-20, Nucleic Acid Chromatography System-20; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

[2] This hydrocarbon moiety is commonly referred to as an isopentenyl unit. However, isopentenyl groups have a C(3)═C(4) in the five-carbon isoprene unit instead of the C(2)═C(3) found in dimethylallyl units.

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A37 plays a central role in the cellular response to certain environmental stresses (Connolly & Winkler, 1991).

Although DMAPP-tRNA transferase has been partially purified from several sources (Bartz et al., 1970; Bartz & Söll, 1972; Fittler et al., 1968; Holtz & Klämbt, 1975, 1978; Kline et al., 1969; Rosenbaum & Gefter, 1972), none of the proteins is well characterized. The *E. coli* enzyme was purified 550-fold and was studied using a coupled isopentenyl diphosphate (IPP) isomerase/DMAPP-tRNA transferase system to generate DMAPP *in situ* for the modification reaction (Rosenbaum & Gefter, 1972). The coupled system required suitably undermodified tRNA, IPP, a divalent cation, and 2-mercaptoethanol for maximal activity. The pH optimum for the coupled system was 7.5. The K_m reported for undermodified $tRNA^{Tyr}$ was 660 nM. The analogous *Saccharomyces cerevisiae* enzyme was purified 50−100-fold, exhibited a pH optimum of 7.5, and required undermodified tRNA, a divalent cation, and 2-mercaptoethanol (Kline et al., 1969). The enzyme had a K_m of 25 μM for a crude yeast tRNA extract.

The lack of homogeneous samples of enzyme and undermodified tRNA has severely hampered biochemical studies of DMAPP-tRNA transferase. The recent isolation and characterization of the *E. coli miaA* gene encoding DMAPP-tRNA transferase and the *pheU* gene for *E. coli* $tRNA^{Phe}$ have provided the necessary tools to address these problems. We now report construction of *E. coli* strains for the synthesis and purification of substantial quantities of recombinant enzyme and undermodified $tRNA^{Phe}$ and the characterization of the recombinant enzyme.

EXPERIMENTAL PROCEDURES

Materials. Plasmid pJO3, containing the *E. coli miaA* gene, was obtained from Professor Joel Caillet (Institut de Biologie Physico-Chimique) (Caillet & Droogmans, 1988). Enzymes were purchased from New England Biolabs, Boehringer Mannheim, or Sigma. pBluescript II SK-(+) was purchased from Stratagene. Taq DNA polymerase was purchased from U.S. Biochemical. Oligonucleotides and the inosine-containing oligoribonucleotide corresponding to the anticodon stem−loop portion of *E. coli* $tRNA^{Phe}$ were synthesized in the Utah Regional Cancer Center Protein/DNA Core Facility. The bacterial expression plasmid pTTQ18 was obtained from Amersham and modified to pTTQ18N, by adding an *Nde*I site that overlaps the ATG codon for initiation of translation. Plasmid pRK3, containing the gene for *E. coli* $tRNA^{Phe}$, was obtained from Professor Bruce Roe (University of Oklahoma) (Schwartz et al., 1983). Phenylalanyl-tRNA synthetase was isolated from *E. coli* IBPC1671/pB1 and purified by the method of Tinkle-Peterson and Uhlenbeck (Plumbridge et al., 1980; Tinkle-Peterson & Uhlenbeck, 1992). Purified *E. coli* $tRNA^{Lys}$ was purchased from Sigma. Benzoylated DEAE-cellulose (BD-cellulose) was from Serva, and NACS-20 resin was from Life Technologies, Inc. Microcrystalline DE52 ion-exchange resin was from Whatmann. [1-^{3}H]DMAPP was from American Radiolabelled Chemicals. DMAPP was synthesized by the method of Davisson et al. (1986). Stock solutions of DMAPP (10 mM) were prepared in 25 mM NH_4HCO_3, and the concentration of DMAPP was determined by phosphate analysis (Reed & Rilling, 1976).

Strains. E. coli strain DH5α was used for all plasmid manipulations. *E. coli* strain JM101 was used for expression of recombinant DMAPP-tRNA transferase. *E. coli* strain CJ236 was purchased from Bio-Rad and used for site-directed mutagenesis experiments. Cells for general cloning and expression were grown in LB media supplemented with ampicillin (100 μg/mL). *E. coli* strain LS1075 (*miaA*$^-$)/pRK3 was used as a source for undermodified $tRNA^{Phe}$ (Eisenberg et al., 1979).

General Procedures. Plasmid preparations, transformations, and other standard molecular biology procedures were carried out as described elsewhere (Sambrook et al., 1989). DNA sequencing was performed by the dideoxy chain termination method on denatured double-stranded templates using the Sequenase kit (U.S. Biochemical). Protein was analyzed by 12% SDS−polyacrylamide gel electrophoresis on a Bio-Rad Mini-PROTEAN II electrophoresis system. Protein bands were stained by Coomassie Brilliant Blue R. Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard (Scopes, 1982). UV measurements were obtained on a Varian Cary 4 UV−visible spectrophotometer. HPLC was performed on a Waters 625 LC system equipped with a Waters 991 photodiode array detector and Rheodyne 9125 injector. Electrospray ionization mass spectra were obtained in the University of Utah Mass Spectrometry Facility by the method of Limbach et al. (1995). Radioactivity was measured in Cytoscint scintillation media (ICN) with a Packard Tricarb Model 2300TR liquid scintillation analyzer.

Construction of an Expression Vector for Production of DMAPP-tRNA Transferase-EEF. Primer-adapted PCR on pJO3 was used to introduce restriction sites at the ends of the *miaA* open reading frame. The sense strand primer (5′-CGGGC*TCTAGA*G**CATATG**AGTGATATCAGT-AAGGCGAGC-3′) introduced a unique *Xba*I restriction site (italics) upstream of the *miaA* start codon (underlined) and an *Nde*I site (bold) that overlapped the ATG initiation codon. The antisense strand primer (5′-CGC**AAGCTT**TCA*GAACTC-CTC*GCCTGCGATAGCACCAACAAC-3′) introduced a unique *Hin*dIII restriction site (bold) downstream of the *miaA* stop codon (underlined) and a mutation which added codons for a C-terminal Glu-Glu-Phe α-tubulin epitope (italics). The amplified 950 bp DNA fragment was ligated into pBluescript II SK-(+) as an *Xba*I−*Hin*dIII cassette (pJAM-I-183) and sequenced to verify the fidelity of the PCR. The orf was excised as an *Nde*I−*Hin*dIII cassette and ligated into *Nde*I−*Hin*dIII-digested pTTQ18N to prepare pJAM-I-212 for expression of *miaA*-containing codons for the C-terminal Glu-Glu-Phe α-tubulin epitope.

Construction of an Expression Vector for Production of Wild Type DMAPP-tRNA Transferase. A plasmid for synthesis of wild type *E. coli miaA* protein was constructed by loopout mutagenesis of the codons for the C-terminal Glu-Glu-Phe α-tubulin epitope in pJAM-I-183 by the method of Kunkel (1985). The mutagenic primer (5′-GTCGACGG-TATCGATAAGAAT**TCA** GCCTGCGATAGCACCAAC-3′) also introduced a unique *Eco*RI restriction site (underlined) immediately downstream of the *miaA* stop codon (bold). The resulting plasmid, pJAM-IV-14, was sequenced to verify the integrity of the mutagenesis experiment. The *miaA* orf was excised as an *Nde*I−*Sal*I cassette and ligated into *Nde*I−*Sal*I-digested pTTQ18N to prepare pJAM-IV-40 for synthesis of wild type *miaA* protein.

Overproduction of Recombinant E. coli DMAPP-tRNA Transferase. Cultures of *E. coli* strain JM101/pJAM-I-212

(or pJAM-IV-40) were grown to an OD_{600} of ~0.5 in LB containing ampicillin (100 μg/mL) at 37 °C with vigorous aeration and then induced with IPTG (0.5 mM final concentration). Incubation was continued for 2.5 h at 37 °C before the cells were harvested by centrifugation (7000*g*, 10 min) and stored at −78 °C.

Purification of Recombinant E. coli DMAPP-tRNA Transferase-EEF. All enzyme isolation procedures were carried out at 4 °C. Frozen cell paste (0.32 g) of *E. coli* strain JM101/pJAM-I-212 was resuspended in 3.5 mL of extraction buffer (50 mM potassium phosphate, 10 mM $MgCl_2$, 10 mM BME, and 10% glycerol at pH 6.5) and disrupted by sonication. The cell-free homogenate was clarified by centrifugation and loaded onto a 1 × 22 cm DE52 cellulose column equilibrated with extraction buffer. The column was washed with extraction buffer and eluted with a 300 mL linear gradient of KCl (0 to 600 mM) in extraction buffer. Recombinant DMAPP-tRNA transferase-EEF eluted at ~200 mM KCl. Active fractions were combined and dialyzed against affinity buffer (50 mM Tris, 10 mM $MgCl_2$, 10 mM BME, 100 mM KCl, and 10% glycerol at pH 7) and loaded onto an anti-α-tubulin immunoaffinity column (10 cm × 0.75 cm) equilibrated in this buffer (Skinner et al., 1991; Stammers et al., 1991). The column was washed with buffer (~25 mL) and then eluted with affinity buffer containing 5 mM Asp-Phe. Fractions containing DMAPP-tRNA transferase-EEF activity were combined, dialyzed against 50 mM Tris, 10 mM $MgCl_2$, 10 mM BME, and 10% glycerol at pH 7, and stored at 0 or −78 °C until needed. The protein isolated from this purification is greater than 95% pure by SDS−PAGE.

Purification of Recombinant E. coli DMAPP-tRNA Transferase. The steps for purification of *E. coli* DMAPP-tRNA transferase from frozen cell paste (1.51 g) of *E. coli* strain JM101/pJAM-IV-40 were identical to those described for DMAPP-tRNA transferase-EEF through DE52 chromatography. Active fractions were pooled, dialyzed against 50 mM Tris, 10 mM $MgCl_2$, 10 mM BME, and 10% glycerol at pH 7, and loaded onto a Pharmacia Mono-Q 5/5 column equilibrated in dialysis buffer. The column was eluted with a 50 mL linear gradient of KCl (0 to 500 mM) in dialysis buffer. Active fractions were pooled, loaded onto a Pharmacia Superdex S200 16/60 column, and eluted with dialysis buffer. DMAPP-tRNA transferase obtained was greater than 95% pure by SDS−PAGE.

Overproduction and Purification of Undermodified $tRNA^{Phe}$. A single colony of freshly transformed *E. coli* strain LS1075/pRK3 was used to inoculate 3 mL of SB medium (15 g of bactotryptone, 10 g of yeast extract, and 5 g of NaCl, all per liter) containing 100 μg/mL ampicillin and 12 μg/mL tryptophan, and the culture was incubated at 37 °C overnight. One milliliter of the overnight culture was diluted into 100 mL of fresh medium, and the cells were grown at 37 °C for 4 h. Ten milliliter aliquots were then used to inoculate 5 × 1 L of fresh medium; the cells were grown for 13 h at 37 °C and then harvested by centrifugation. Cell paste (43 g) was homogenized in 75 mL denaturing/lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 100 mM BME at pH 7), and RNA was extracted by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski & Sacchi, 1987). RNA was precipitated from the aqueous layer with an equal volume of 2-propanol, and the extraction/precipitation process was repeated. Precipitated RNA was washed with 70% EtOH, resuspended in 4 M LiCl, and allowed to stand at −20 °C for 2 h. Insoluble material was removed by centrifugation, and tRNA was precipitated from the supernatant with an equal volume of 2-propanol at −20 °C. The precipitate was redissolved in 8 mL of BD-cellulose chromatography buffer (20 mM NaOAc, 10 mM $MgCl_2$, and 0.4 M NaCl at pH 6) and dialyzed against this buffer. The sample was loaded in two portions onto a 1.5 × 24 cm BD-cellulose column and eluted with a 500 mL linear gradient of NaCl (0.4 to 1.5 M). Fractions containing $tRNA^{Phe}$ (assayed for [^{3}H]Phe acceptor activity) were combined, precipitated with 2-propanol, redissolved in 5 mL of NACS buffer (10 mM NaOAc, 10 mM $MgCl_2$, and 0.2 M NaCl at pH 4.5), and dialyzed against this buffer. The sample was loaded onto a 1.5 × 10 cm NACS-20 column and eluted with a 500 mL linear gradient of NaCl (0.2 to 1.0 M). Fractions containing $tRNA^{Phe}$ were precipitated with 2-propanol, redissolved in NACS buffer, dialyzed as above, and rechromatographed on the NACS-20 column. The final yield was 0.5−1 mg/L of culture (823 A_{260} units mM^{-1} cm^{-1} for pure $tRNA^{Phe}$) (Curnow et al., 1993).

DMAPP-tRNA Transferase Assay. DMAPP-tRNA transferase activity was assayed by a modified TCA-precipitation procedure (Igloi et al., 1979). $tRNA^{Phe}$ was heat annealed just prior to use by placing samples in a 75 °C water bath which was allowed to cool gradually to 37 °C. Enzyme was diluted to the appropriate concentration with 50 mM Tris/BSA (1 mg/mL) at pH 7.5. A standard assay mixture contained 50 mM Tris (pH 7.5), 3.5 mM $MgCl_2$, 5 mM BME, 7.7 μM $tRNA^{Phe}$, 50 μM [^{3}H]DMAPP (25 Ci/mol), 1 mg/mL BSA, and ~20 ng of enzyme in a total volume of 50 μL. The reaction was initiated by addition of enzyme to a pre-equilibrated assay mixture (37 °C, 5 min), and incubation was continued for 2 min at 37 °C. Assays were quenched by the addition of 25 μL of cold 10% TCA. Each assay mixture was spotted on a Whatmann GF/C filter and allowed to air-dry. The filters were washed twice for 10 min with 10% TCA at 0 °C (10 mL/filter), twice for 10 min with 95% EtOH, and finally with diethyl ether. After drying, the samples were mixed with 10 mL of Cytoscint, and radioactivity was determined by liquid scintillation spectrometry. Assays were run in duplicate or triplicate. Control assays were run in an identical manner except that enzyme dilution buffer or fully modified bulk tRNA was substituted for enzyme or undermodified $tRNA^{Phe}$, respectively.

K_M for DMAPP was measured from initial velocities obtained in the standard assay buffer at a saturating concentration of $tRNA^{Phe}$ (7.7 μM), different fixed concentrations of DMAPP (1, 2.5, 5, 10, 20, and 30 μM), and 10 nM enzyme. K_M for $tRNA^{Phe}$ was measured from initial velocities obtained at a saturating concentration of DMAPP (10 μM) and 50, 75, 100, 150, 250, 500, and 1000 nM $tRNA^{Phe}$.

Assays to determine the K_i value for the inosine-containing $tRNA^{Phe}$ minihelix were carried out as described above except that the minihelix was heat annealed in the presence of $MgCl_2$ (3.5 mM final concentration in assay) by heating at 80 °C for 3 min and rapidly cooling on ice. The assay mixtures contained 50 mM Tris (pH 7.5), 3.5 mM $MgCl_2$, 5 mM BME, 75−750 μM $tRNA^{Phe}$, 4 μM [^{3}H]DMAPP (1000 Ci/mol), 1 mg/mL BSA, 0.5−4 μM minihelix, and ~3 ng of enzyme in a total volume of 50 μL.

Product Analysis. Reaction mixtures (50 μL) were incubated at 37 °C for 1 h in assay buffer containing 12.8 μM tRNA^{Phe} and 75 μM DMAPP. Purified DMAPP-tRNA transferase (366 ng, 5 μL) was added three times at 20 min intervals. A control reaction was identical except that DMAPP was omitted. Reactions were terminated by dilution to 200 μL with DEPC-treated H_2O. The mixture was extracted once with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and once with $CHCl_3$/isoamyl alcohol (24:1). tRNA^{Phe} in the aqueous extract was purified on NACS-20 minicolumns (Life Technologies, Inc.) according to the manufacturer's protocol and digested to its individual nucleosides by the method of Gehrke et al., except that the tRNA was incubated with 3 units of nuclease P1 for 15 h followed by addition of 15 μL of 0.5 M Tris (pH 8.5), 2 μL of 20 mM $ZnSO_4$, and 0.5 unit of bacterial alkaline phosphatase and incubation for 3 h (Gehrke et al., 1982). The composition of the modified bases from digested tRNA^{Phe} was analyzed by HPLC on a Varian SP-C18-5 (4 × 15 cm) reversed-phase column. The column was eluted at 1 mL/min with a linear gradient from 0 to 40% CH_3CN in H_2O (pH 3 with H_3PO_4) over 50 min. Elution profiles were recorded at 254 nm.

Native Molecular Mass Determination. Samples of purified DMAPP-tRNA transferase were chromatographed on a Pharmacia Superdex 75 HR 10/10 column. The column was calibrated with alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), pepsin (34.7 kDa), and cytochrome *c* (12.4 kDa). DMAPP-tRNA transferase activity was determined by the TCA-precipitation assay, as previously described.

Fluorescence Titration of tRNA^{Phe} Binding to the Enzyme. The binding of undermodified tRNA^{Phe} to DMAPP-tRNA transferase was monitored by the change in the intrinsic fluorescence intensity of the enzyme. Reaction mixtures (200 μL) contained 50 mM Tris (pH 7.5), 3.5 mM $MgCl_2$, 5 mM BME, 21.4 nM enzyme, and 0−150 nM tRNA^{Phe}. Fluorescence measurements were obtained in 3 mm square cuvettes using a Spex Fluoromax model spectrofluorimeter with excitation and emission wavelengths of 295 and 340 nm, respectively, and bandwidths of 5 nm. Background emission was corrected for by subtracting the fluorescence value at ∞ tRNA^{Phe} concentration determined from a graph of ΔF versus [tRNA^{Phe}].

Measurement of the Dissociation Constant for DMAPP. The binding of DMAPP to an inosine-containing tRNA^{Phe} minihelix inhibitor−enzyme binary complex was measured by the method of Dolence et al. (1995). Briefly, DMAPP-tRNA transferase (1 μg, 300 nM) was added to [^{3}H]DMAPP (450 Ci/mol, 0.4−15 μM) in 50 mM Tris, 3.5 mM $MgCl_2$, 5 mM BME, and 10 μM inosine-containing minihelix at pH 7.5, and the mixtures were incubated at 37 °C for 15 min. A sample (10 μL) was removed and mixed with 5 mL of Cytoscint to determine the total radioactivity of the sample. The remainder of the mixture was transferred to a pre-equilibrated Micron-10 (Amicon) and concentrated at 14 000 rpm in a microcentrifuge for 30 s or until approximately 10−15 μL of the mixture had passed through the membrane. A 10 μL portion of the filtrate was removed and mixed with 5 mL of Cytoscint to determine the concentration of free substrate. The radioactivity was corrected for membrane retention using a correction factor obtained from an experiment identical except that enzyme was omitted, and the data were then fit to a standard binding equation to obtain K_D and the number of binding sites per mole of enzyme (Fersht, 1985).

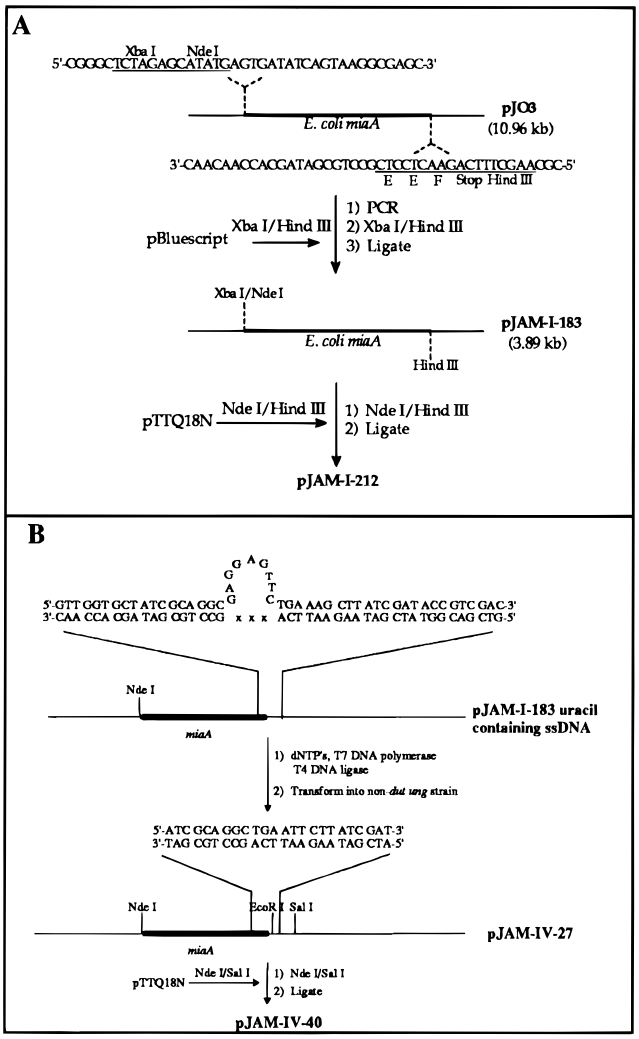

FIGURE 1: Construction of expression vectors for synthesis of (A) DMAPP-tRNA transferase-EEF and (B) wild type DMAPP-tRNA transferase.

RESULTS

Cloning and Expression of E. coli miaA. The orf for *E. coli miaA* was amplified from pJO3 by PCR-mediated site-directed mutagenesis (Figure 1). The PCR product contained a unique *Xba*I site 9 bp upstream of the translation initiation codon, a unique *Nde*I site within the translation initiation codon, codons for a C-terminal Glu-Glu-Phe tripeptide, and a unique *Hin*dIII site immediately downstream of the translation termination codon. The amplified DNA was ligated into pBluescript II SK-(+) as an *Xba*I−*Hin*dIII cassette (pJAM-I-183) and sequenced to ensure that no unwanted mutations had occurred. The *miaA* open reading frame was excised as an *Nde*I−*Hin*dIII cassette and placed under the control of a *tac* promoter in pTTQ18N to give expression plasmid pJAM-I-212. A plasmid that directed synthesis of wild type DMAPP-tRNA transferase was generated from pJAM-I-183 by loopout mutagenesis of the codons encoding the C-terminal Glu-Glu-Phe tripeptide. The primer also introduced a two-base mutation (AG to TT) at position two of the unique *Hin*dIII site immediately downstream of the translation termination codon to generate a

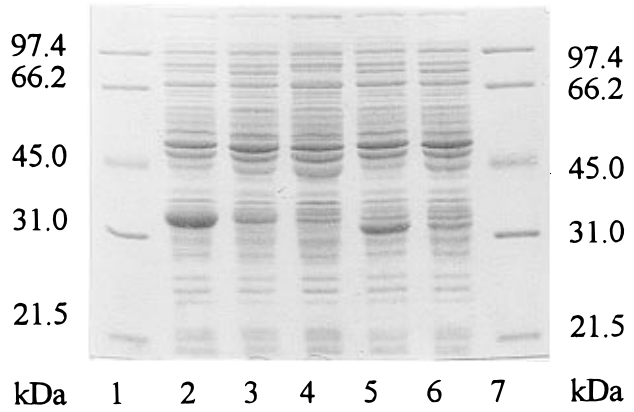

FIGURE 2: SDS gel of crude cell-free extracts prepared from *E. coli* transformants JM101/pJAM-I-212 and *E. coli* JM101/pJAM-IV-40: lane 1, molecular mass markers; lane 2, crude extract from induced *E. coli* strain JM101/pJAM-I-212; lane 3, crude extract from uninduced *E. coli* strain JM101/pJAM-I-212; lane 4, crude extract from *E. coli* strain JM101/pTTQ18N; lane 5, crude extract from induced *E. coli* strain JM101/pJAM-IV-40; lane 6, crude extract from uninduced *E. coli* strain JM101/pJAM-IV-40; and lane 7, molecular mass markers.

Table 1: Purification of Recombinant Wild Type DMAPP-tRNA Transferase

steps	protein (mg)	units (μmol/min)	yield (%)	purification (-fold)	specific activity (μmol min^{-1} mg^{-1})
crude extract	75.4	10.6	100	1	0.14
DE52	9.6	4.5	42	3.4	0.47
Mono-Q	7.4	6.0	57	5.8	0.81
Superdex S200	3.7	4.3	41	8.2	1.15

unique *Eco*RI site within the translation termination codon. The *miaA* open reading frame was excised as an *Nde*I−*Sal*I cassette and placed under the control of a *tac* promoter in pTTQ18N, generating expression plasmid pJAM-IV-40.

Transformants of *E. coli* strain JM101 containing either pJAM-I-212 or pJAM-IV-40 were grown to midlog phase, induced with IPTG, and incubated for an additional 2.5 h. Analysis of cell-free extracts prepared from *E. coli* strains JM101/pJAM-I-212 or pJAM-IV-40 by SDS−PAGE showed new proteins that migrated with apparent molecular masses of 34.7 and 33.4 kDa, respectively (Figure 2). These proteins were not seen in SDS gels of cell-free extracts prepared from the untransformed host strain or from cultures of uninduced *E. coli* strain JM101/pJAM-I-212 or pJAM-IV-40 (Figure 2).

Purification of DMAPP-tRNA transferase. Recombinant wild type *E. coli* DMAPP-tRNA transferase was purified 8-fold to >95% homogeneity by a combination of ion-exchange (DE52 and Mono-Q) and size exclusion chromatography. The protocol, summarized in Table 1, provided material which gave a single band at ~34.7 kDa by SDS−PAGE. Similarly, recombinant DMAPP-tRNA transferase-EEF was purified 16-fold to >95% homogeneity by ion exchange on DE52 and immunoaffinity chromatography on an anti-α-tubulin immunoaffinity column (Skinner et al., 1991; Stammers et al., 1991). The protocol for purification of DMAPP-tRNA transferase-EEF is summarized in Table 2. Acrylamide−SDS gels of samples from each stage of the purifications are shown in Figure 3. The recombinant

Table 2: Purification of Recombinant DMAPP-tRNA Transferase-EEF

steps	protein (mg)	units (μmol/min)	yield (%)	purification (-fold)	specific activity (μmol min^{-1} mg^{-1})
crude extract	9.5	0.86	100	1	0.09
DE52	1.3	0.56	65	5.1	0.43
affinity purified	0.16	0.22	27	16.5	1.40

enzymes are stable for several months at −78 °C and for longer than 1 week at 0 °C.

Overproduction and Purification of E. coli tRNA^{Phe}. Undermodified tRNA^{Phe} required for studies of DMAPP-tRNA transferase was purified from *E. coli* strain LS1075/pRK3, an *miaA*$^-$ mutant transformed with a multicopy plasmid containing *pheU*, the gene for *E. coli* tRNA^{Phe} (Davis & Poulter, 1991; Schwartz et al., 1983; Wilson & Roe, 1989). The levels of tRNA^{Phe} in the transformed host strain were approximately 13-fold higher than that in an untransformed control, as estimated from HPLC traces (Figure 4A,B). Undermodified tRNA^{Phe} was purified by acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski & Sacchi, 1987), BD-cellulose chromatography, and two rounds of NACS-20 chromatography (Tanner, 1989; Thompson et al., 1983). tRNA^{Phe} isolated in this manner eluted as a single peak at ~19 min from a Phenomenex W-POREX C4 reversed-phase HPLC column eluted with a 60 mL descending, linear gradient of $(NH_4)_2SO_4$ (1.0 to 0 M) in 100 mM potassium phosphate and 0.75% 2-propanol at pH 7 (Figure 4C) (Pearson et al., 1983).

The reconstructed molecular mass of purified tRNA^{Phe} from an electrospray mass spectrum corresponds to a molecular mass of 24 700 ± 1.2 Da. This value is consistent with the predicted molecular mass of 24 697 Da for *E. coli* tRNA^{Phe} lacking the 2-methylthio and dimethylallyl groups at A37.

Recombinant DMAPP-tRNA Transferase Product Analysis. The starting and final reaction mixtures from incubation of DMAPP, undermodified tRNA^{Phe}, and recombinant DMAPP-tRNA transferase were analyzed by HPLC on a C4 reversed-phase column and showed that the band for the undermodified tRNA had disappeared with the concomitant formation of a new peak for the modified tRNA (data not shown). Reversed-phase HPLC was then used to analyze the nucleosides released from tRNA^{Phe} upon hydrolysis by nuclease P1 and dephosphorylation of the resultant nucleotides with bacterial alkaline phosphatase (Figure 5). i^{6}A is considerably more hydrophobic than other nucleosides and appeared at a distinct position late in the gradient (26 min, Figure 5A). A peak with the same retention time as an authentic sample of i^{6}A was observed in the hydrosylate derived from tRNA^{Phe} incubated with recombinant enzyme and DMAPP (Figure 5B). Undermodified tRNA^{Phe} incubated with recombinant enzyme in the absence of DMAPP did not have a peak corresponding to i^{6}A in the HPLC chromatogram of its hydrosylate (Figure 5C).

General Properties and Kinetic Constants of Recombinant DMAPP-tRNA Transferase. The native molecular mass of the recombinant DMAPP-tRNA transferase was measured on a calibrated Superdex 75 column (data not shown). Values of 37.8 and 33.7 kDa were obtained for DMAPP-tRNA transferase-EEF and DMAPP-tRNA transferase, re-

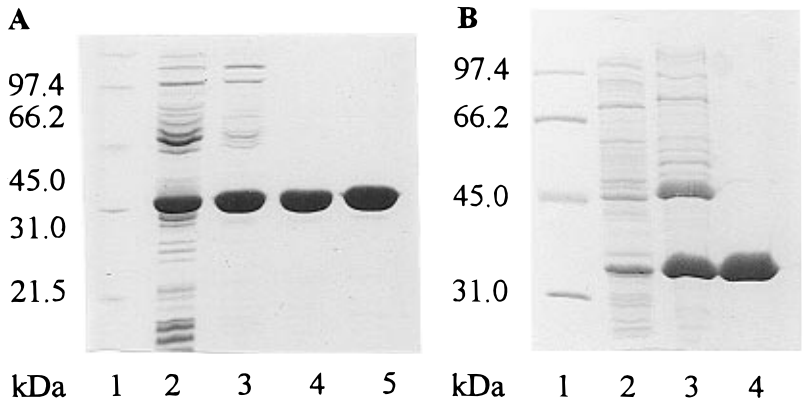

FIGURE 3: Purification of recombinant DMAPP-tRNA transferase. (A) A 12% SDS gel of proteins from each step in the purification of wild type DMAPP-tRNA transferase: lane 1, molecular mass standards; lane 2, crude extract from *E. coli* strain JM101/pJAM-IV-40; lane 3, after DE52 chromatography; lane 4, after Mono-Q chromatography; and lane 5, after Superdex S200 chromatography. (B) A 12% SDS−PAGE of proteins from each step in the purification of DMAPP-tRNA transferase-EEF: lane 1, molecular mass standards; lane 2, crude extract from *E. coli* strain JM101/pJAM-I-212; lane 3, after DE52 chromatography; and lane 4, after immunoaffinity chromatography.

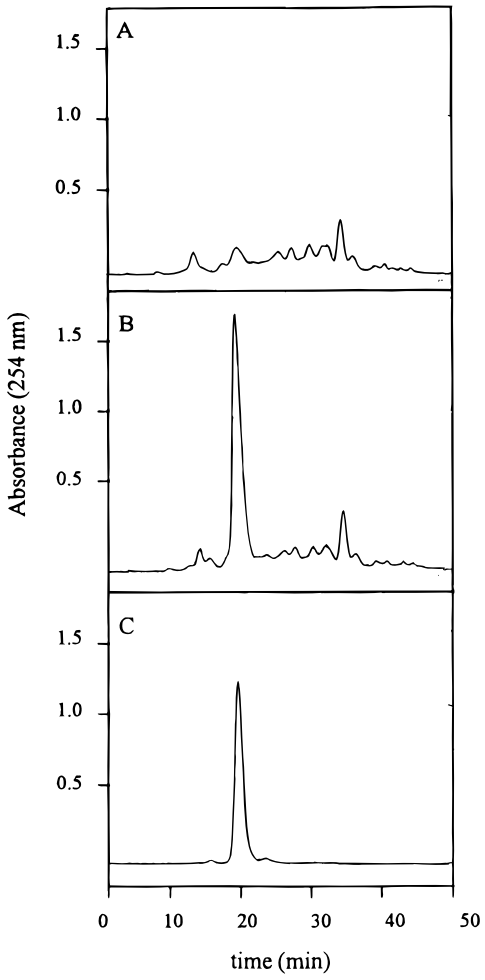

FIGURE 4: HPLC analysis for tRNA: (A) crude extract from *E. coli* strain LS1075, (B) crude extract from *E. coli* LS1075/pRK3, and (C) purified $tRNA^{Phe}$.

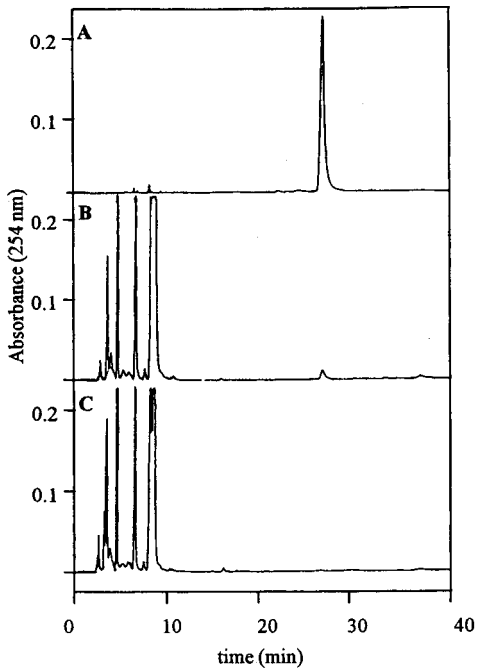

FIGURE 5: HPLC analysis of nucleosides contained in $tRNA^{Phe}$: (A) an i^6A standard obtained from Sigma, (B) nucleoside content of $tRNA^{Phe}$ after reaction with DMAPP-tRNA transferase and DMAPP, and (C) nucleoside content in $tRNA^{Phe}$ after reaction with DMAPP-tRNA transferase without DMAPP.

spectively. The recombinant enzyme is inactive when assayed in metal-free buffer after dialysis in the presence of EDTA, and activity is restored upon addition of Mg^{2+} to an optimal level of ~1 mM (Figure 6A). The pH rate profile of DMAPP-tRNA transferase measured in a Tris/MES/acetic acid polybuffer is a broad "bell-shaped" curve with a maximum between 6.5 and 9 (Figure 6B).

Initial velocities were measured for varied concentrations of one substrate in the presence of a saturating concentration of the other (7.7 μM $tRNA^{Phe}$ or 11.6 μM DMAPP) in the standard assay buffer. Michaelis constants were determined by explicit or simple weighted nonlinear regression analysis utilizing GraFit (Leatherbarrow, 1992). The K_m values obtained for $tRNA^{Phe}$ and DMAPP were 96 ± 11 nM and 3.2 ± 0.5 μM, respectively, and $k_{cat} = 0.48\ s^{-1}$.

Dissociation Constants for E. coli $tRNA^{Phe}$ and DMAPP. The association of DMAPP-tRNA transferase with undermodified $tRNA^{Phe}$ was monitored by the change in the intrinsic fluorescence intensity of the enzyme. Titration of the enzyme with increasing concentrations of $tRNA^{Phe}$ resulted in a quenching of protein fluorescence to a maximum value of about 35%. A plot of ΔF versus [$tRNA^{Phe}$] yielded a hyperbolic curve (Figure 7) from which ΔF_{max}, the fluorescence change when all enzyme molecules are complexed, was determined. The background for DMAPP-tRNA transferase fluorescence was determined by subtracting ΔF_{max} from the intrinsic enzyme fluorescence measured without

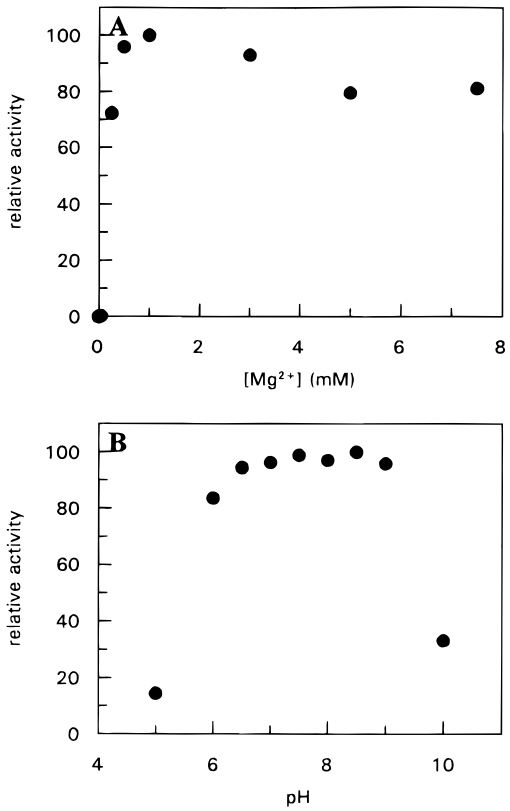

FIGURE 6: Relative reaction velocities obtained at (A) different concentrations of $MgCl_2$ and (B) at different pHs. Samples of DMAPP-tRNA transferase for studies of the Mg^{2+} ion dependence of the reaction were first dialyzed against 50 mM Tris, 10 mM EDTA, 10 mM BME, and 10% glycerol at pH 7.5 and then against same buffer without EDTA. All assays contained 50 mM Tris (pH 7.5), 5 mM BME, 7.7 μM $tRNA^{Phe}$, 50 μM [^{3}H]DMAPP (25 Ci/mol), and ~13 ng of enzyme. The pH rate profiles were obtained using a polybuffer between pH 5 and 10 containing 100 mM Tris, 50 mM MES, 50 mM acetic acid, 3.5 mM $MgCl_2$, 5 mM BME, 7.7 μM $tRNA^{Phe}$, 50 μM [^{3}H]DMAPP (25 Ci/mol), and ~19 ng of enzyme.

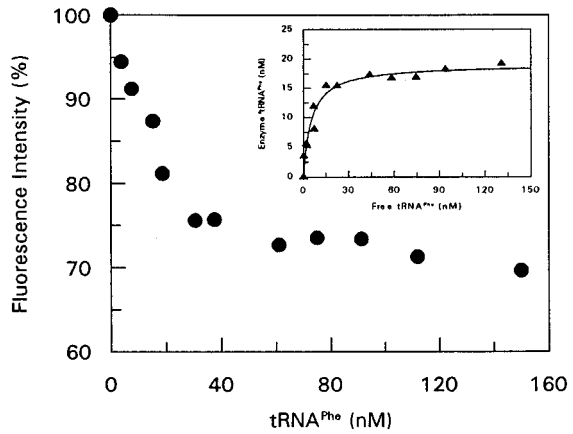

FIGURE 7: Fluorescence titration of DMAPP-tRNA transferase with undermodified $tRNA^{Phe}$. (Inset) A plot of bound versus free substrate for the determination of the dissociation constant for $tRNA^{Phe}$.

added $tRNA^{Phe}$ and was subtracted from the fluorescence measured at each $tRNA^{Phe}$ concentration to give a corrected value. Bound and free $tRNA^{Phe}$ were calculated from eqs 1 and 2, where E_t, corrected F_{obs}, and corrected $^0F_{obs}$ refer to the total enzyme, the corrected fluorescence measurement at each [$tRNA^{Phe}$], and the corrected fluorescence measurement without added $tRNA^{Phe}$, respectively.

$$\text{bound } tRNA^{Phe} = E_t - (\text{corrected } F_{obs})(E_t/\text{corrected } {}^0F_{obs}) \quad (1)$$

$$\text{free } tRNA^{Phe} = \text{total } tRNA^{Phe} - \text{bound } tRNA^{Phe} \quad (2)$$

The data were then fitted to a standard binding equation (Fersht, 1985) to obtain K_D and the number of binding sites per mole of enzyme (Figure 7, inset). With 21.4 nM DMAPP-tRNA transferase, values for K_D of 5.2 ± 1.2 nM and a capacity of 19.0 ± 0.9 nM, or 0.89 binding site per mole of enzyme, were obtained. In a control experiment, no substantial fluorescence quenching was seen with *E. coli* $tRNA^{Lys}$, a tRNA with substantial sequence similarity to *E. coli* $tRNA^{Phe}$.

A membrane filter assay was used in an effort to determine the dissociation constant for an enzyme−DMAPP complex (Dolence et al., 1995). After the correction for membrane retention of [^{3}H]DMAPP, determined from control reactions in which the enzyme was omitted, free [^{3}H]DMAPP was subtracted from total [^{3}H]DMAPP to determine the concentration of enzyme-bound substrate. The allylic substrate did not bind to the enzyme at concentrations up to 20 μM DMAPP. In contrast, the affinity of DMAPP-tRNA transferase for DMAPP increased dramatically in the presence of a nonreactive tRNA minihelix analogue. A 17-mer minihelix, corresponding to the anticodon stem−loop region of $tRNA^{Phe}$ containing an inosine at the prenylation site (A37), inhibited the DMAPP-tRNA transferase reaction with a K_i value of 2.7 ± 0.6 μM (Figure 8). In solutions containing 10 μM inosine-containing minihelix and 300 nM DMAPP-tRNA transferase, $K_D = 3.4 \pm 0.6$ μM for DMAPP, and the enzyme had a capacity of 197 ± 12 nM, corresponding to 0.66 binding site per mole.

DISCUSSION

E. coli DMAPP-tRNA transferase catalyzes the first step in the biosynthesis of the hypermodified residue A37 in tRNAs that read codons beginning with uridine. The reaction results in the transfer of a dimethylallyl moiety to the amino group of A37, providing i^6A. A methylthio group is added at C(2) of A37 in a subsequent Fe^{2+}, cysteine, and SAM-dependent reaction to complete the hypermodification. In *E. coli*, i^6A-modified tRNAs include those specific for Phe, Tyr, Ser, Leu, Trp, and Cys.

Undermodified $tRNA^{Phe}$ was isolated and purified from an *E. coli miaA* mutant strain LS1075/pRK3 containing the gene for $tRNA^{Phe}$ on a multicopy plasmid. *E. coli* $tRNA^{Phe}$ lacking only the 2-methylthio and dimethylallyl groups on A37 has a predicted molecular mass of 24 697 Da. Electrospray mass spectral analysis of the purified $tRNA^{Phe}$ confirmed that the sample was homogeneous and contained all modifications except those normally found at A37. Thus, the other modifications found in $tRNA^{Phe}$ occur independently of the i^6A modification, and prenylation of A37 is required before the remaining modifications can proceed at that residue. An electrospray mass spectrum obtained on a $tRNA^{Phe}$ sample that had been modified by recombinant DMAPP-tRNA transferase was more complex. A reconstructed molecular mass peak of 24 765 Da corresponding to $tRNA^{Phe}$ that had been modified with a dimethylallyl group was observed. However, several higher-molecular mass peaks were also present, and the signal to noise ratio was

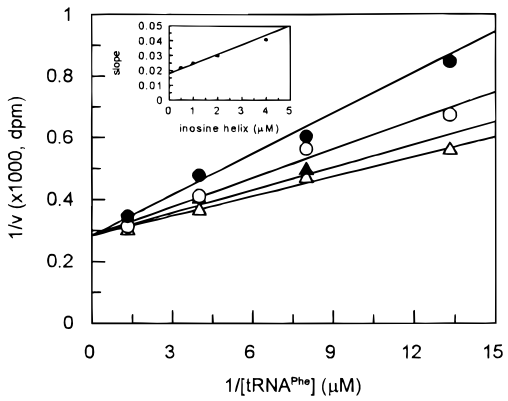

FIGURE 8: Double-reciprocal plot with $tRNA^{Phe}$ as the varied substrate at fixed concentrations of the inosine-containing $tRNA^{Phe}$ minihelix. Concentrations of inosine-containing $tRNA^{Phe}$ minihelix were 0.5 (△), 1 (▲), 2 (○), and 4 (●) μM. The concentration of DMAPP was 4 μM. (Inset) Slopes versus the concentration of the inosine-containing $tRNA^{Phe}$ minihelix.

not optimal. The higher-molecular mass peaks and the poor signal to noise ratio may have been due to incomplete removal of Mg^{2+} from the tRNA sample prior to mass spectral analysis.

Wild type and an affinity-tagged DMAPP-tRNA transferase containing a C-terminal Glu-Glu-Phe epitope were purified from *E. coli* strain JM101 containing expression plasmids where the orfs for the proteins were under control of a tac promoter. The C-terminal Glu-Glu-Phe epitope in the affinity-tagged enzyme is specifically recognized by YL1/2 monoclonal antibodies to anti-α-tubulin. Purification is facilitated by immunoaffinity chromatography on a column of immobilized YL1/2 antibodies through highly selective binding of the protein containing the Glu-Glu-Phe motif. Highly purified recombinant DMAPP-tRNA transferase-EEF was eluted with an Asp-Phe-containing buffer after extensive washing to remove weakly bound proteins. The recombinant wild type and EEF-tagged enzymes were identical in all aspects examined except for the slightly larger molecular mass exhibited by the affinity-tagged protein on SDS gels and size exclusion columns.

The apparent molecular mass of DMAPP-tRNA transferase determined by density gradient centrifugation of a partially purified sample was reported to be 55 kDa (Bartz et al., 1970). The size of the *E. coli miaA* protein predicted from the gene sequence is ∼35 kDa, which prompted Connolly and Winkler (1991) to suggest that the enzyme might be a homodimer. Purified recombinant DMAPP-tRNA transferase elutes from a calibrated Superdex 75 size exclusion column with an apparent molecular mass of ∼34 kDa. Thus, we conclude that the active enzyme is monomeric.

Previous kinetic studies of DMAPP-tRNA transferase were conducted by coupling IPP isomerase and DMAPP-tRNA transferase (Rosenbaum & Gefter, 1972). [^{3}H]DMAPP required for prenylation of the tRNA substrate, which was not available commercially, was generated *in situ* from [^{3}H]-IPP by the action of porcine liver IPP isomerase. The design of this assay made it difficult to separate the properties of DMAPP-tRNA transferase from those of the coupled system. Porcine liver IPP isomerase independently requires Mg^{2+}, a thiol reagent, and exhibits a pH optimum of 6.3 (Banthorpe et al., 1977), while the coupled system reportedly had a pH optimum of 7.5 and a Mg^{2+} optimum of 3.3 mM (Rosenbaum & Gefter, 1972). We found a broad pH optimum for DMAPP-tRNA transferase with a plateau that extended from pH 6.5 to 9. The recombinant enzyme also required Mg^{2+} for activity, with a broad maximum extending from ∼1 to 8 mM Mg^{2+}. The Michaelis constant for $tRNA^{Phe}$ (K_m = 96 ± 11 nM) obtained in this study is lower than that reported for $tRNA^{Tyr}$ (K_m = 0.66 μM) using the coupled assay. Previous studies with the coupled assay system precluded the determination of a Michaelis constant for DMAPP. We obtained a value of 3.2 ± 0.5 μM.

Scheme 1: Proposed Minimal Mechanism for DMAPP-tRNA Transferase

$$\text{Enz} + \text{tRNA}^{\text{Phe}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Enz}\cdot\text{tRNA}^{\text{Phe}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Enz}\cdot\text{tRNA}^{\text{Phe}}\cdot\text{DMAPP} \xrightarrow{k_3} \text{products}$$

(DMAPP binds at k_2)

The binding of $tRNA^{Phe}$ and DMAPP to DMAPP-tRNA transferase was investigated by a combination of fluorescence titration and membrane filter assay experiments. Fluorescent residues in proteins include the aromatic rings in tryptophan, tyrosine, and phenylalanine, with fluorescence being dominated in most proteins by the tryptophan residues. The fluorescence λ_{max} for tryptophan is reported to be 348 nm, while the fluorescence λ_{max}'s for tyrosine and phenylalanine are 303 and 282 nm, respectively. In a series of exploratory experiments, we discovered that $tRNA^{Phe}$ quenched the intrinsic fluorescence of the enzyme at 340 nm. Thus, we believe the binding of $tRNA^{Phe}$ to DMAPP-tRNA transferase results in a fluorescence quenching of a tryptophan residue(s). A maximum fluorescence quenching of ∼35% was achieved when all enzyme was complexed with $tRNA^{Phe}$. This is similar to quenching profiles observed for other tRNA binding enzymes (Bhattacharyya et al., 1991; Lin et al., 1988). Conversely, DMAPP did not quench the intrinsic fluorescence of the enzyme nor could any binding of DMAPP alone be detected using the membrane filter assay. However, DMAPP did bind to the enzyme in the presence of a nonreactive $tRNA^{Phe}$ analogue corresponding to the anticodon stem−loop region of $tRNA^{Phe}$ with an inosine in place of A37 at the prenylation site. The inosine-containing minihelix is a competitive inhibitor with respect to $tRNA^{Phe}$ with a K_i of 2.7 ± 0.6 μM. In the presence of the inosine-containing minihelix (10 μM), the membrane filter assay yielded values for K_D of 3.4 ± 0.6 μM for DMAPP and 0.66 binding site per mole of enzyme. The number of binding sites is lower than expected and might arise from less than saturating levels of the inosine-containing minihelix in the assays or from uncertainties in the enzyme concentration as determined by the Bradford assay.

A minimal binding mechanism consistent with these observations is the ordered sequential process shown in Scheme 1, where the prenyl acceptor ($tRNA^{Phe}$) binds first, followed by the allylic substrate (DMAPP). The extremely tight binding of the tRNA substrate, the lack of binding by the allylic substrate in the absence of tRNA, and the restoration of DMAPP binding in the presence of a nonreactive tRNA stem−loop require that the tRNA substrate bind first. This binding order stands in contrast to that of other prenyltransferase enzymes for which the binding mechanism has been determined. Protein farnesyltransferase (PFTase) from yeast (Dolence et al., 1995) and mammalian sources (Furfine et al., 1995; Pompliano et al., 1993) and avian liver farnesyl diphosphate synthase (FPP synthase) (Laskovics et

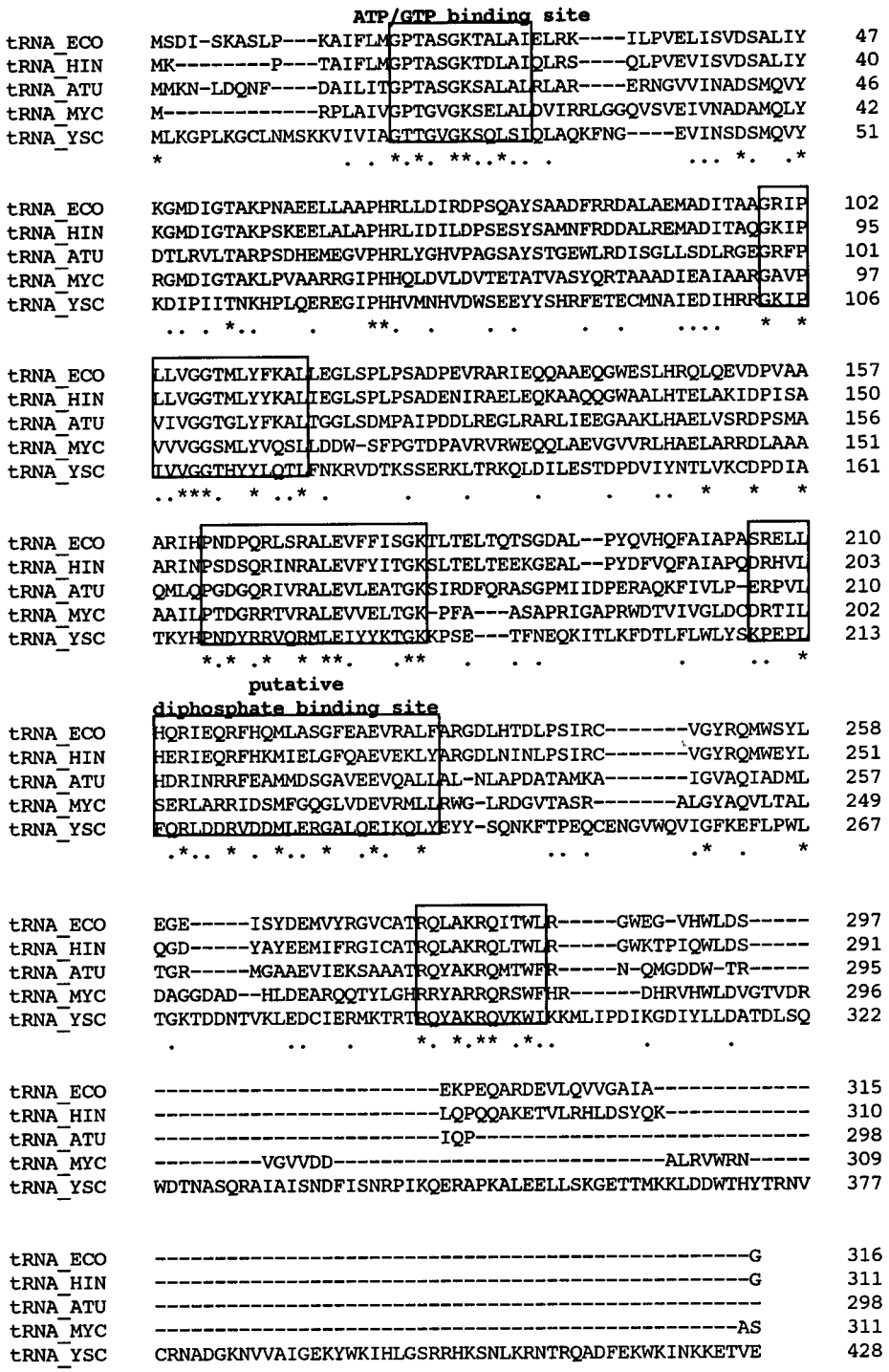

FIGURE 9: Multiple sequence alignment for five DMAPP-tRNA transferases: tRNA_ECO, *E. coli*; tRNA_HIN, *H. influenzae* Rd; tRNA_ATU, *A. tumefaciens*; tRNA_MYC, *M. leprae*; and tRNA_YSC, *S. cerevisiae*.

al., 1979) also bind substrates by an ordered sequential mechanism, but for these enzymes, the allylic substrate adds first, followed by the prenyl acceptor.

The complete DNA sequences have now been identified for the *mia*A genes from *E. coli*, *A. tumefaciens*, *Haemophilus influenzae* Rd, and *Mycobacterium leprae* and the *MOD5* gene in *S. cerevisiae*, encoding DMAPP-tRNA transferases (Connolly & Winkler, 1991; Fleischmann et al., 1995; Gray et al., 1992; Najarian et al., 1987). A fragment of the *mia*A gene in *Saccharomyces typhimurium* also has been identified (Mankovich et al., 1989; Robison et al., 1994). Thirteen additional complete DNA sequences have been identified for *ipt*, *tmr*, *tzs*, and *ptz* genes all encoding DMAPP-AMP transferases (Akiyoshi et al., 1985, 1989; Beaty et al., 1986; Bonnard et al., 1989; Canaday et al., 1992; Crespi et al., 1992; Goldberg et al., 1984; Heidkamp et al., 1983; Otten & De Ruffray, 1994; Powell & Morris, 1986; Regier et al., 1989; Strabala et al., 1989; A. L. Lichter, I. B. Barash, L. V. Valinsky, and S. M. Manulis, unpublished data). DMAPP-AMP transferases catalyze the transfer of a dimethylallyl moiety to the amino group of 5′-AMP in a reaction identical to the DMAPP-tRNA transferase-catalyzed reaction. Translation of these nucleotide sequences and alignment of the deduced protein sequences using CLUSTAL (PCGENE) show that the DMAPP-AMP transferases are all smaller (~27 kDa) than the prokaryotic DMAPP-tRNA

transferases (~34 kDa). The yeast DMAPP-tRNA transferase is the largest enzyme (~50 kDa) and contains a C-terminal extension of unknown significance not found in the bacterial enzymes. A high degree of sequence similarity is seen throughout the DMAPP-tRNA transferases with five highly conserved regions found throughout the proteins (Figure 9). Four of these regions were identified earlier from an alignment of the *E. coli*, *A. tumefaciens*, and yeast DMAPP-tRNA transferases by Gray et al. (1992). One of the highly conserved regions includes a putative ATP/GTP binding site close to the amino terminus of the five proteins (amino acids 17−27 in the *E. coli* enzyme). The ATP/GTP binding site consists of a consensus sequence for a nucleotide binding fold seen in distantly related sequences from ATP synthase, myosin, kinases, and other ATP-requiring enzymes (Walker et al., 1982). Interestingly, ATP or GTP is not required for DMAPP-tRNA transferase activity. The nucleotide binding fold may play an important role in the recognition of A37 in the anticodon region of prenylated tRNAs. *E. coli* $tRNA^{Phe}$ contains an adenosine at position 37 and is bound tightly by the enzyme ($K_D = 5.2 \pm 1.2$ nM). In contrast, *E. coli* $tRNA^{Lys}$ which has a high degree of sequence identity (60%) to $tRNA^{Phe}$ but does not contain an adenosine at position 37 is not a substrate and is bound very weakly by DMAPP-tRNA transferase. A putative DMAPP binding domain (amino acids 206−234 in the *E. coli* enzyme) was originally assigned in the yeast protein by comparison with other prenyltransferases. Several of these enzymes have isoprenoid diphosphate binding domains consisting of aspartate rich motifs with a DDXXD consensus sequence, where X can be any amino acid (Ashby & Edwards, 1990). The conserved aspartate residues are thought to facilitate the binding of isoprenoid diphosphate substrates through magnesium salt bridges between the negatively charged carboxylates of aspartic acid and the diphosphate ligands. Although a DDXXD diphosphate binding domain is located in the fourth conserved region of *S. cerevisiae* DMAPP-tRNA transferase, the aspartate rich motif is only found in the yeast protein, and further studies are required to locate the diphosphate binding site. Sequence similarities among all the DMAPP-AMP transferases (*ipt*, *tmr*, *tzs*, and *ptz* gene products) are also high. However, sequence similarities between the DMAPP-AMP transferases and the DMAPP-tRNA transferases are less obvious. The only highly conserved region found throughout all the sequences is the putative ATP/GTP binding site close to the amino terminus of the proteins.

Finally, the nomenclature used to denote i^6A as a Δ^2-isopentenyladenosine is archaic. Isopentenyl normally denotes a 3-methyl-3-butenyl unit, while the 3-methyl-2-butenyl moiety attached to adenosine is a dimethylallyl unit. Thus, the modified base is a (dimethylallyl)adenosine derivative, and the modifying enzymes are (dimethylallyl)transferases.

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REFERENCES

Akiyoshi, D. E., Regier, D. A., Jen, G., & Gordon, M. P. (1985) *Nucleic Acids Res. 13*, 2773−2788.
Akiyoshi, D. E., Regier, D. A., & Gordon, M. P. (1989) *Nucleic Acids Res. 17*, 8886.
Ashby, M. N., & Edwards, P. A. (1990) *J. Biol. Chem. 265*, 13157−13164.
Banthorpe, D. V., Doonan, S., & Gutowski, J. A. (1977) *Arch. Biochem. Biophys. 184*, 381−390.
Bartz, J. K., & Söll, D. (1972) *Biochemie 54*, 31−39.
Bartz, J. K., Kline, L. K., & Söll, D. (1970) *Biochem. Biophys. Res. Commun. 40*, 1481−1487.
Beaty, J. S., Powell, G. K., Lica, L., Regier, D. A., MacDonald, E. M. S., Hommes, N. G., & Morris, R. O. (1986) *Mol. Gen. Genet. 203*, 274−280.
Bhattacharyya, T., Bhattacharyya, A., & Roy, S. (1991) *Eur. J. Biochem. 200*, 739−745.
Blum, P. H. (1988) *J. Bacteriol. 170*, 5125−5133.
Bonnard, G., Tinland, B., Paulus, F., Szegedi, E., & Otten, L. (1989) *Mol. Gen. Genet. 216*, 428−438.
Buck, M., & Griffiths, E. (1981) *Nucleic Acids Res. 9*, 401−414.
Buck, M., & Griffiths, E. (1982) *Nucleic Acids Res. 10*, 2609−2624.
Buck, M., & Ames, B. N. (1984) *Cell 36*, 523−531.
Buck, M., McCloskey, J. A., Basile, B., & Ames, B. N. (1982) *Nucleic Acids Res. 10*, 5649−5662.
Caillet, J., & Droogmans, L. (1988) *J. Bacteriol. 170*, 4147−4152.
Canaday, J., Gerad, J. C., Crouzet, P., & Otten, L. (1992) *Mol. Gen. Genet. 235*, 292−303.
Cherayil, J. D., & Lipsett, M. N. (1977) *J. Bacteriol. 131*, 741−744.
Chomczynski, P., & Sacchi, N. (1987) *Anal. Biochem. 162*, 156−159.
Connolly, D. M., & Winkler, M. E. (1991) *J. Bacteriol. 173*, 1711−1721.
Crespi, M., Messens, E., Caplan, A. B., van Monagu, M., & Desomer, J. (1992) *EMBO J. 11*, 795−804.
Curnow, A. W., Kung, F.-L., Koch, K. A., & Garcia, G. A. (1993) *Biochemistry 32*, 5239−5246.
Davis, D. R., & Poulter, C. D. (1991) *Biochemistry 30*, 4223−4231.
Davisson, V. J., Woodside, A. B., Neal, T. R., Stremler, K. E., Muehlbacher, M., & Poulter, C. D. (1986) *J. Org. Chem. 51*, 4768−4779.
Dolence, J. M., Cassidy, P. B., Mathis, J. R., & Poulter, C. D. (1995) *Biochemistry 34*, 16687−16694.
Eisenberg, S. P., Yarus, M., & Söll, L. (1979) *J. Mol. Biol. 135*, 111−126.
Fersht, A. (1985) in *Enzyme Structure and Mechanism*, Chapter 6, W. H. Freeman and Co., New York.
Fittler, F., Kline, L. K., & Hall, R. H. (1968) *Biochem. Biophys. Res. Commun. 31*, 571−576.
Fleischmann, R. D., et al. (1995) *Science 269*, 496−512.
Furfine, E. S., Leban, J. J., Landavazo, A., Moomaw, J. F., & Casey, P. J. (1995) *Biochemistry 34*, 6857−6862.
Gefter, M. L., & Russell, R. L. (1969) *J. Mol. Biol. 39*, 145−157.
Gehrke, C. W., Kuo, K. C., McCune, R. A., Gerhardt, K. O., & Agris, P. F. (1982) *J. Chromatogr. 230*, 297−308.
Goldberg, S. B., Flick, J. S., & Rogers, S. G. (1984) *Nucleic Acids Res. 12*, 4665−4677.
Gray, J., Wang, J., & Gelvin, S. B. (1992) *J. Bacteriol. 174*, 1086−1098.
Heidkamp, F., Dirkse, W. G., Hille, J., & Van ormondt, H. (1983) *Nucleic Acids Res. 11*, 6211−6223.
Holtz, J., & Klämbt, D. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 1459−1464.
Holtz, J., & Klämbt, D. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 89−101.
Igloi, G. L., Von der Haar, F., & Cramer, F. (1979) *Methods Enzymol. 59*, 282−291.
Janzer, J. J., Raney, J. P., & McLennan, B. D. (1982) *Nucleic Acids Res. 10*, 5663−5672.
Kersten, H. (1984) *Prog. Nucleic Acid Res. Mol. Biol. 31*, 59−114.
Kline, L. K., Fittler, F., & Hall, R. H. (1969) *Biochemistry 8*, 4361−4371.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
Laskovics, F. M., Krafcik, J. M., & Poulter, C. D. (1979) *J. Biol. Chem. 254*, 9458−9463.

Laten, H., Gorman, J., & Bock, R. M. (1978) *Nucleic Acids Res. 5*, 4329−4342.
Leatherbarrow, R. J. (1992) *Grafit*, Version 3.01, Erithicus Software Ltd., Staines, U.K.
Limbach, P. A., Crain, P. F., & McCloskey, J. A. (1995) *J. Am. Soc. Mass Spectrom. 6*, 27−39.
Lin, S. X., Wang, Q., & Wang, Y. L. (1988) *Biochemistry 27*, 6348−6353.
Mankovich, J. A., McIntyre, C. A., & Walker, G. C. (1989) *J. Bacteriol. 171*, 5325−5331.
Najarian, D., Dihanich, M. E., Martin, N. C., & Hopper, A. K. (1987) *Mol. Cell. Biol. 7*, 185−191.
Otten, L., & De Ruffray, P. (1994) *Mol. Gen. Genet. 245*, 493−505.
Pearson, J. D., Mitchell, M., & Regnier, F. E. (1983) *J. Liq. Chromatogr. 6*, 1441−1457.
Plumbridge, J. A., Springer, M., Graffe, M., Goursot, R., & Grunberg-Manago, M. (1980) *Gene 11*, 33−42.
Pompliano, D. L., Schaber, M. D., Mosser, S. D., Omer, C. A., Shafer, J. A., & Gibbs, J. B. (1993) *Biochemistry 32*, 8341−8347.
Powell, G. K., & Morris, R. O. (1986) *Nucleic Acids Res. 14*, 2555−2565.
Reed, B. C., & Rilling, H. C. (1976) *Biochemistry 15*, 3739−3745.
Regier, D. A., Akiyoshi, D. E., & Gordon, M. P. (1989) *Nucleic Acids Res. 17*, 8885.
Robison, K., Gilbert, W., & Church, G. M. (1994) *Nat. Genet. 7*, 205−214.
Rosenbaum, N., & Gefter, M. L. (1972) *J. Biol. Chem. 247*, 5675−5680.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
Schwartz, I., Klotsky, R. A., Elseviers, D., Gallagher, P. J., Krauskopf, M., Siddiqui, M. A. Q., Wong, J. F. H., & Roe, B. A. (1983) *Nucleic Acids Res. 11*, 4379−4389.
Scopes, R. (1982) in *Protein Purification Principles and Practice*, p 266, Springer-Verlag, Berlin.
Skinner, R. H., Bradley, S., Brown, A. L., Johnson, N. J. E., Rhodes, S., Stammers, D. K., & Lowe, P. N. (1991) *J. Biol. Chem. 266*, 14163−14166.
Stammers, D. K., Tisdale, M., Court, S., Parmar, V., Bradley, C., & Ross, C. K. (1991) *FEBS Lett. 283*, 298−302.
Strabala, T. J., Bednarek, S. Y., Bertoni, G., & Amasino, R. M. (1989) *Mol. Gen. Genet. 216*, 388−394.
Tanner, N. K. (1989) *Methods Enzymol. 180*, 25−41.
Thimmappaya, B., & Cherayil, J. D. (1974) *Biochem. Biophys. Res. Commun. 60*, 665−672.
Thompson, J. A., Blakesley, R. W., Doran, K., Hough, C. J., & Wells, R. D. (1983) *Methods Enzymol. 100*, 368−399.
Tinkle-Peterson, E., & Uhlenbeck, O. C. (1992) *Biochemistry 31*, 10380−10389.
Walker, J. E., Saraste, M., Runswick, M. J., & Gay, N. J. (1982) *EMBO J. 1*, 945−951.
Wilson, R. K., & Roe, B. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 409−413.

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