Escherichia coli Dimethylallyl Diphosphate:tRNA Dimethylallyltransferase: Site-Directed Mutagenesis of Highly Conserved Residues†

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ABSTRACT: Dimethylallyl diphosphate:tRNA dimethylallyltransferase (DMAPP-tRNA transferase) catalyzes alkylation of the exocyclic amine of adenosine at position 37 in some tRNAs by the hydrocarbon moiety of dimethylallyl diphosphate (DMAPP). A multiple-sequence alignment of 28 gene sequences encoding DMAPP-tRNA transferases from various organisms revealed considerable homology, including 11 charged, 12 polar, and four aromatic amino acids that are highly conserved or conservatively substituted. Site-directed mutants were constructed for all of these amino acids, and a tripeptide Glu-Glu-Phe α-tubulin epitope was appended to the C-terminus of the protein to facilitate separation by immunoaffinity chromatography of overproduced mutant enzymes from coexpressed chromosomally encoded wild-type DMAPP-tRNA transferase. Steady-state kinetic constants were measured for wild-type DMAPP-tRNA transferase and the site-directed mutants using DMAPP and a 17-base RNA oligoribonucleotide corresponding to the stem−loop region of $tRNA^{Phe}$ as substrates. Substantial changes in k_{cat}, K_m^{DMAPP}, and/or K_m^{RNA} were seen for several of the mutants, suggesting possible roles for these residues in substrate binding and catalysis.

Transfer RNAs undergo a variety of post-transcriptional modifications that are important for their biological activity (*1*, *2*). Although modified bases are found at different positions in tRNAs, the anticodon loop is the most heavily modified region of the molecule and contains the greatest variety of modified nucleotides. N^6-(Dimethylallyl)adenosine (i^6A)[1] is commonly found at position 37 of those tRNAs that also have an adenosine at position 36, the last anticodon base (see Figure 1 for the numbering scheme). The formation of i^6A involves transfer of the dimethylallyl moiety from dimethylallyl diphosphate (DMAPP) to the amino group in adenosine, with concurrent release of inorganic pyrophosphate. In many organisms, A37 is further modified by addition of a thiomethyl group to C2, followed by hydroxylation at C_5 of the dimethylallyl group.

The function of the i^6A modification has been studied extensively. The presence of the dimethylallyl group does not appear to significantly alter the kinetics of aminoacylation (*3*, *4*) and is not required for viability of bacterial or yeast hosts (*5*, *6*). However, the dimethylallyl modification improves binding of tRNA to the ribosome during translation (*4*) through enhanced stacking interactions in the codon−anticodon complex, thus stabilizing the relatively weak A·U base pairing between A36 and the uridine of the codon (*7*, *8*). Other studies indicate that a wide range of cellular processes are affected by the presence of i^6A, including the expression of genes involved in amino acid biosynthesis (*9*) and bacterial virulence (*10*), aromatic amino acid uptake (*11*), growth on citric acid cycle intermediates (*12*), and cellular response to environmental stress (*13*).

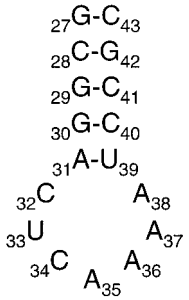

FIGURE 1: Seventeen-base RNA oligoribonucleotide used as a tRNA substrate analogue in this study.

Dimethylallyl diphosphate:tRNA dimethylallyltransferase (DMAPP-tRNA transferase) catalyzes alkylation of the amine moiety in adenosine by the five-carbon dimethylallyl unit in DMAPP. Recently, two separate groups purified the recombinant *Escherichia coli* protein to homogeneity (*14*, *15*). DMAPP-tRNA transferase is a monomer. Steady-state kinetic studies with recombinant enzyme suggest an ordered sequential addition of substrates, where the tRNA substrate binds before dimethylallyl diphosphate (DMAPP) (*14*, *16*). The normal tRNA substrates can be replaced with a 17-base pair oligoribonucleotide minihelix corresponding to the anticodon stem−loop fragment of $tRNA^{Phe}$ (Figure 1). When $tRNA^{Phe}$ was replaced with the minihelix in the enzyme-catalyzed reaction, k_{cat} and K_M^{DMAPP} were unchanged, but K_M^{tRNA} increased 180-fold (*17*). Pre-steady-state experiments

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[1] Abbreviations: BME, β-mercaptoethanol; DMAPP, dimethylallyl diphosphate; DMAPP-tRNA transferase, dimethylallyl diphosphate:tRNA dimethylallyltransferase; i^6A, N^6-(dimethylallyl)adenosine; IPTG, isopropyl thio-β-D-galactoside; PMSF, phenylmethanesulfonyl chloride; TGT, tRNA-guanine transglycosylase.

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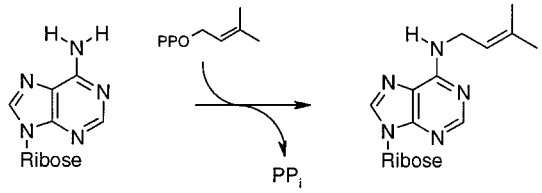
(*16*) showed that enzyme-bound DMAPP is in a rapid equilibrium with free DMAPP and that the rate-limiting step for turnover is the chemical conversion of the substrate to product. Studies with a series of stem−loop RNA oligoribonucleotide substrates containing single and double base substitutions revealed that the enzyme has a broad selectivity for its tRNA substrates. The only stringent requirements for recognition were an A36-A37-A38 motif adjacent to a region of helical secondary structure (*17*).

Scheme 1: Modification of Adenosine by DMAPP



Multiple sequence alignments of the proteins encoded by DMAPP-tRNA transferase genes from a wide variety of organisms show several regions with conserved charged, polar, and aromatic amino acids (Figure 2). A completely conserved GxxxGKT(S) motif closely resembles the so-called "P-loop", a well-characterized nucleotide di- and triphosphate binding motif (*18*−*21*). The P-loop unit is also found in dimethylallyl diphosphate:adenosine-5′-monophosphate transferase, an enzyme of the cytokinin biosynthetic pathway in plants (*22*). An arginine-rich motif in the tRNA transferase is similar to the conserved RxxR sequence in tRNA-guanine transglycosylase (TGT), an enzyme required for the queuine modification at position 34 (*23*). However, the amino acid sequences of DMAPP-tRNA transferases do not closely resemble those of other isoprenoid prenyltransferases, including protein farnesyltransferases (*24*, *25*), dimethylallyltryptophan synthases (*26*, *27*), farnesyl diphosphate synthases (*28*−*31*), and archaebacterial geranylgeranylglyceryl diphosphate synthase (*32*) or closely related terpene cyclases (*33*−*35*), nor does DMAPP-tRNA transferase have the DDxxD diphosphate-binding motif found in many prenyltransferases (*28*−*31*, *33*−*35*). We describe here mutagenesis studies of several highly conserved amino acids in DMAPP-tRNA transferase to identify those important for substrate binding and catalysis.

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                aI   S     S a   a        aa       aW
MSDISKASLP KAIFLMGPTA SGKTALAIEL RKILPVELIS VDSALIYKGM 50

 V S R        a                    r          a
DIGTAKPNAE ELLAAPHRLL DIRDPSQAYS AADFRRDALA EMADITAAGR 100

  Va   S
IPLLVGGTML YFKALLEGLS PLPSADPEVR ARIEQQAAEQ GWESLHRQLQ 150

             N         QI   T
EVDPVAAARI HPNDPQRLSR ALEVFFISGK TLTELTQTSG DALPYQVHQF 200

r                        a                        a
AIAPASRELL HQRIEQRFHQ MLASGFEAEV RALFARGDLH TDLPSIRCVG 250

  E    r                 S    R K   F
YRQMWSYLEG EISYDEMVYR GVCATRQLAK RQITWLRGWE GVHWLDSEKP 300

EQARDEVLQV VGAIAG 316
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FIGURE 2: Amino acid sequence of DMAPP-tRNA transferase from *E. coli*. Bold residues represent those that are conserved or conservatively substituted. Underlined residues are those for which mutations produced large changes in enzyme kinetic parameters. Letters above residues specify conservative substitutions that are present in other DMAPP-tRNA transferases. The letter "a" is used for aliphatic residues (alanine, valine, leucine, and isoleucine), "r" for aromatic residues (phenylalanine, tyrosine, and tryptophan), and "b" when aliphatic and aromatic substitutions are seen.

MATERIALS AND METHODS

Materials. Plasmid pJAM I-212, which contains the *miaA* gene from *E. coli* with an engineered C-terminal Glu-Glu-Phe tripeptide epitope to facilitate purification of the encoded protein by immunoaffinity chromatography, was available from previous studies (*14*). Addition of the tripeptide did not alter the catalytic properties of the enzyme significantly. Oligonucleotide primers for mutagenesis and RNA oligoribonucleotide substrates were synthesized by the Protein/DNA Sequencing Core Facility at the Huntsman Cancer Institute at the University of Utah. The GeneEditor kit from Promega was used for all site-directed mutagenesis. DE52 ion-exchange resin was purchased from Whatman, and Gamma-bind Plus resin for immunoaffinity chromatography was from Amersham-Pharmacia. DMAPP was synthesized by the method of Davisson et al. (*36*), and stock solutions were prepared in 25 mM NH_4HCO_3. DMAPP concentrations were determined by phosphate analysis (*37*). [1-^{3}H]DMAPP (specific activity = 22 Ci/mmol) was purchased from NEN Life Science Products.

Strains. E. coli strain DH5α was used for routine DNA manipulations. *E. coli* strain BMH 71-18 *mutS* was used as part of the GeneEditor mutagenesis kit (Promega). *E. coli* strain JM101 was used to produce the recombinant protein.

General Procedures. Plasmid preparations were performed with the Wizard Plus Miniprep system from Promega and the Midi Plasmid kit from Qiagen. DNA sequencing was performed by the Protein/DNA Sequencing Core Facility at the Huntsman Cancer Institute at the University of Utah. Protein was analyzed by 12% SDS−PAGE on a Bio-Rad Mini-PROTEAN II electrophoresis system and visualized with Coomassie Brilliant Blue R.

Putative DMAPP-tRNA transferases from 28 organisms were identified from the nonredundant GenBank database using the BLAST search algorithm, and were compared in a multiple-sequence alignment. The organisms and GenBank accession numbers are as follows: *Agrobacterium tumefaciens* (M83532), *Aquifex aeolicus* (AE000721), *Arabidopsis thaliana* (AF109376), *Bacillus subtilis* (Z99113), *Borrelia burgdorferi* (AE001180), *Caenorhabditis elegans* (U13642), *Campylobacter jejuni* (CAB72649), *Chlamydia muridarum* (AAF39024), *Chlamydia trachomatis* (AE001273), *Chlamydophila pneumoniae* (AE001671), *Deinococcus radiodurans* (AAF11245), *E. coli* (AE000489), *Haemophilus influenzae* (U32692), *Helicobacter pylori* (AE001554), *Mycobacterium leprae* (U00019), *Mycobacterium tuberculosis* (AL123456), *Neisseria meningitidis* (AAF41341), *Pseudomonas putida* (AF016312), *Rhodobacter capsulatus* (RRC03576), *Rickettsia prowazekii* (AJ235272), *Saccharomyces cerevisiae* (M15991), *Schizosaccharomyces pombe* (AL109739), *Shigella flexneri* (AB000785), *Streptomyces coelicolor* (AL022268), *Synechocystis* sp. (AB001339), *Thermotoga maritima* (AE001728), *Treponema pallidum* (AE001238), and *Zymomonas mobilis* (AF176314).

Mutagenesis was carried out on plasmid pJAM I-212 with the GeneEditor kit from Promega according to the manufacturer's protocols. Mutations were confirmed by DNA sequencing.

Synthesis and Purification of Wild-Type (wt)[2] *and Mutant E. coli DMAPP-tRNA Transferases.* Enzymes were produced in *E. coli* JM101 transformants upon induction by IPTG as previously described (*14*). The proteins were purified by a slightly modified version of the procedure of Moore and Poulter (*14*). Frozen cell paste was resuspended in buffer A [50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 10 mM BME, and 10% glycerol] containing 1 mM PMSF, and disrupted by sonication. The cell-free extract was clarified by centrifugation and loaded onto a 2 cm × 20 cm DE52 cellulose column equilibrated with buffer A, and the column was washed with 150 mL of the same buffer. Protein was eluted with buffer B (buffer A with 150 mM KCl), and fractions containing DMAPP-tRNA transferase, as determined by SDS−PAGE analysis, were loaded directly onto a 0.75 cm × 18 cm anti-α-tubulin affinity column equilibrated with buffer B. The column was washed with 30 mL of buffer B, and then eluted with buffer C (buffer B with 5 mM Asp-Phe). Fractions containing protein were dialyzed overnight against 1 L of buffer A and stored at −80 °C. The wild-type and mutant proteins purified in this manner gave single bands upon SDS−PAGE, and the yields were similar to those obtained in previous work (*14*).

DMAPP-tRNA Transferase Activity Assay. DMAPP-tRNA transferase activity was assayed as described previously (*14*), except that reaction volumes were reduced from 50 to 20 μL. A 17-base oligoribonucleotide with the sequence 5′GCGGACUCAAAAUCCGC3′, based on the unmodified stem−loop region of $tRNA^{Phe}$, was used as the tRNA substrate (Figure 1). The RNA was diluted in 10 mM Tris, 1 mM EDTA, and 3.5 mM $MgCl_2$ (pH 8.0), placed in boiling water for 5 min, and then quickly cooled on ice for 30 min before being used. Previous studies demonstrated that the RNA oligonucleotide anneals in a stem−loop secondary structure similar to that of the stem−loop region of tRNA under these conditions (*17*). Values of K_m^{DMAPP} for the wild-type enzyme and mutants were measured from initial velocities at a fixed concentration of RNA, typically 50 μM. K_m^{RNA} was measured from initial velocities at fixed DMAPP concentrations of 10−100 μM, depending on the properties of the enzyme. Reaction times were typically 5−10 min. Steady-state kinetic constants were determined by nonlinear regression analysis using Grafit (*38*).

CD Measurements. CD spectra were recorded on an AVIV 62ADS spectrometer between 200 and 260 nm at intervals of 1 nm. Samples were diluted to 0.5 mL, and the spectra were measured in a 0.5 cm path length quartz cuvette at 20 °C. Spectra of wild-type and mutant proteins were recorded in 50 mM Tris (pH 7.5), 1 mM BME, and 3.5 mM $MgCl_2$. Sample concentrations ranged from 0.05 to 0.15 mg/mL. Three scans were recorded and averaged.

RESULTS

Construction of DMAPP-tRNA Transferase Mutants. Figure 2 shows the amino acid sequence of *E. coli* DMAPP-tRNA transferase with the conserved or conservatively substituted residues identified in a multiple-sequence alignment highlighted in bold type. Of this group, the 28 with ionic, polar, or aromatic side chains typically found in the active sites of prenyltransferases and related enzymes (*25*, *32*−*35*) were selected for substitution by site-directed mutagenesis as indicated in Table 1. Mutagenesis was performed directly on plasmid pJAM I-212 used to produce wt DMAPP-tRNA transferase. The 5′-phosphorylated antisense mutagenic primers contained one or two base mismatches and a 10−15-base pair clamp on either side of the mutation. *E. coli* JM101 cells were transformed with mutagenized plasmids, and protein synthesis was induced by addition of IPTG when the cells reached mid-log phase. The mutant proteins contained a C-terminal Glu-Glu-Phe epitope and were readily purified by immunoaffinity chromatography. This step cleanly resolved the mutant proteins from chromosomally encoded wt DMAPP-tRNA transferase.

Table 1: DMAPP-tRNA Transferase Mutants Generated in This Study[a]

charged	polar	aromatic
D42A	T19A (S)	Y47S
D153A	T24A (S)	Y47F
E229A	S43A	Y111S
K23A	T54A (S)	Y111F
K56A (R)	T108A (S)	F84A (Y,W)
R167A	S178A (T)	W285A (F)
R170A	T275A (S)	
R213A	H67F	
R217A	D164A (N)	
K280A (R)	E173A (Q)	
R281A (K)	Q253A (E)	
	Q282A	

[a] Letters in parentheses indicate conservative substitutions in the sequence alignment.

Characterization of wt and Mutant DMAPP-tRNA Transferases. The steady-state kinetic constants k_{cat}, K_m^{DMAPP}, and K_m^{RNA} were determined from initial velocity measurements for the wt and mutant enzymes, using a 17-base RNA oligoribonucleotide stem−loop minihelix as the RNA substrate (Figure 1). Clarified *E. coli* cell-free extracts from strains producing mutant and wild-type protein were assayed for DMAPP-tRNA transferase activity using 10 μM DMAPP and 20 μM RNA. Seventeen of the mutants had reduced levels of prenyltransferase activity relative to the wt enzyme. These proteins were purified and analyzed further. The results for the mutant enzymes are presented in Table 2 relative to wt DMAPP-tRNA transferase. In some cases, the mutations increased K_m for one or both substrates substantially, and it was not possible to determine the kinetic constants because of limitations on substrate availability or solubility. For the mutants where K_m for one or both of the substrates was too high to calculate k_{cat}, we report v_i^{rel}, the initial velocity (v_i) of the mutant relative to v_i for the wt enzyme measured with 50 μM DMAPP and 50 μM RNA. In those cases where substrate inhibition by DMAPP was observed, v_i was measured at the DMAPP concentration where the enzyme was most active. In all cases, substrate concentrations were at least 10-fold higher than enzyme concentrations, and conditions were adjusted so that less than 10% of the limiting substrate was consumed. For the least active mutants, v_i was measured at less than a single turnover.

Analysis of Site-Directed Mutants. (1) Mutations with No Substantial Effects. A number of the mutations did not significantly alter the activity of DMAPP-tRNA transferase.

[2] DMAPP-tRNA transferase with a C-terminal EEF tripeptide epitope appended to the wild-type enzyme is termed wt DMAPP-tRNA transferase.

Table 2: Steady-State Kinetic Constants for Mutant DMAPP-tRNA Transferases[a]

protein	K_m^{RNA} (μM)	K_m^{DMAPP} (μM)	k_{cat} (s^{-1})	v_i^{rel}
wt	3.2 ± 0.3	2.2 ± 0.2	0.19 ± 0.05	1
T19A	<25	<25	3×10^{-4}	
K23A	>50[c]	>500[c]	*b*	1×10^{-3}
T24A	>50[c]	>500[c]	*b*	6×10^{-4}
D42A	2.8 ± 0.3	2.9 ± 0.3	$(9 \pm 2) \times 10^{-3}$	
Y47F	4.4 ± 0.5	3.5 ± 0.3	0.23 ± 0.03	
Y47S	8.9 ± 0.9	1.4 ± 0.2	$(2 \pm 0.5) \times 10^{-3}$	
T54A	12 ± 2	13 ± 2	$>7 \times 10^{-2}$ [d]	
K56A	50 ± 5	10 ± 5[e]	*b*	0.1
H67F	1.5 ± 0.2	50 ± 5	0.18 ± 0.05	
T108A	16 ± 2	22 ± 3	$(7 \pm 2) \times 10^{-2}$	
D164A	27 ± 3	27 ± 3	>0.15[d]	
R167A	75 ± 8	>25[e]	*b*	3×10^{-2}
R170A	>100[c]	>25[e]	*b*	6×10^{-3}
R217A	7.2 ± 0.8	114 ± 12	$(7 \pm 1) \times 10^{-3}$	
E229A	>50[c]	10 ± 5[e]	*b*	5×10^{-2}
Q253A	56 ± 6	2.3 ± 0.7[e]	*b*	0.3
K280A	>100[c]	75 ± 10	*b*	1×10^{-4}
Q282A	45 ± 5	60 ± 6	>0.3[d]	

[a] For those mutants with large increases in K_m for one or more substrates, the relative initial velocity (v_i^{rel}) is reported. In these cases, v_i was determined using each substrate at 50 μM, or 50 μM RNA and the optimal DMAPP concentration when DMAPP substrate inhibition was observed. [b] Not determined. [c] v_i was linear up to the highest substrate concentrations that were used. [d] Saturating substrate concentrations were not achieved. [e] Substrate inhibition was observed; v_i was measured at the optimal substrate concentration.

Cell-free homogenates containing the D153A, R213A, R281A, Y47F, Y111S, Y111F, F84A, W285A, S43A, S178A, T275A, and E173A proteins all had transferase activities that were at least 25% of that of a similar preparation of the wt enzyme when assayed with 10 μM DMAPP and 20 μM RNA. Levels of production of soluble mutant proteins were similar to that of the wild-type protein as determined by SDS−PAGE analysis of cell-free extracts, and the substrate concentrations for the assay were selected so that a substantial increase in K_m of either substrate would be reflected in a lower activity for the mutant.

(2) Mutations Affecting k_{cat}. Three of the mutations resulted in 10−1000-fold reductions in k_{cat} with little change in the K_m for either substrate, suggesting that the altered amino acids are important for catalysis. The largest change in k_{cat} was observed for T19A, which was approximately 600-fold lower than that of wt DMAPP-tRNA transferase. The D42A mutation resulted in a 20-fold reduction in k_{cat}. Mutation of the conserved Y47 to serine gave a 100-fold lower k_{cat}; however, the steady-state kinetic constants for Y47F, including k_{cat}, were essentially the same as that of the wt enzyme, indicating that the aromatic character of the side chain rather than the hydroxyl group is critical. The sequence for the putative DMAPP-tRNA transferase from *Rh. capsulatus* (ORF 3576) has a tryptophan at this position (see the web page of the *Rh. capsulatus* sequencing project at http://rhodoL.uchicago.edu/capsulapedia/capsulapedia/capsulapedia.shtml).

(3) Mutations Affecting K_m^{RNA}. Three mutations significantly increased K_m for the RNA substrate, and the enzymes exhibited significant substrate inhibition by DMAPP. K_m^{RNA} for K56A was 15-fold larger than for the wt. Initial rates for E229A varied linearly with RNA concentration up to 50 μM, suggesting that K_m^{RNA} is substantially higher. K_m^{DMAPP} for the K56A and E229A enzymes was similar to that of wt DMAPP-tRNA transferase, but both were inhibited at higher DMAPP concentrations. The activities of the E229A and K56A proteins were approximately 5 and 10% of the wild-type value when incubated with 50 μM RNA and 10 μM DMAPP. An alanine mutation at Q253, where glutamate or glutamine is conserved, resulted in an 18-fold increase in K_m^{RNA}. K_m^{DMAPP} for Q253A was similar to that for the wt, but DMAPP concentrations of >25 μM were inhibitory.

(4) Mutations Affecting K_m^{DMAPP}. K_m^{DMAPP} for the H67F mutant was 25-fold higher than for the wt enzyme, while K_m^{RNA} and k_{cat} were essentially unchanged, suggesting that the side chain of H67 interacts with the allylic substrate. The R217A mutation also produced a large increase in K_m^{DMAPP}, as well as a reduction in k_{cat} to 4% of the wt value. These results most likely reflect interactions between the positively charged guanidinium moiety and the phosphate oxygens of DMAPP that are important for binding the isoprenoid substrate and stabilizing inorganic pyrophosphate as it departs.

(5) Mutations Affecting K_m^{RNA} and K_m^{DMAPP}. Some mutations produced large increases in values of K_m for both substrates. The values of K_m for the K23A, T24A, R167A, R170A, and K280A enzymes were so high that saturating substrate concentrations could not be reached in the assays. DMAPP-tRNA transferase contains a conserved GxxxxGKT-(S) motif, which corresponds to a widely conserved phosphate binding motif present in many NTP-binding proteins (*18*). K23 and T24 correspond to the "KT" dyad in the putative "P-loop" motif in *E. coli* DMAPP-tRNA transferase, and both are essential. The initial rates for the K23A and T24A enzymes varied linearly with DMAPP concentrations up to 500 μM (more than 200 times greater than the K_m^{DMAPP} for wt DMAPP-tRNA transferase). The apparent values of K_m for RNA were 10−20 times higher than the wild-type values. Rates measured for the K23A and T24A mutants at a concentration of 50 μM for each substrate were 3 orders of magnitude lower than the wt enzyme rate.

Alanine substitutions at positions 167 and 170 of the *E. coli* sequence, where arginine and lysine are conserved, substantially reduced the catalytic efficiency of the mutants through changes in K_m for both substrates. The K_m^{RNA} for R167A was 20-fold higher than for the wt, while the rate for the R170A enzyme varied linearly with RNA concentration up to 100 μM. When assayed at 50 μM RNA, values of K_m^{DMAPP} for both mutants were at least 10-fold higher than that of the wt enzyme. Both enzymes were inhibited by DMAPP at concentrations of >50 μM. The K280A mutant also exhibited altered kinetic behavior. The rate varied linearly with RNA concentration up to 150 μM, and K_m^{DMAPP} is more than 30 times higher than that of the wt. When assayed with a concentration of 50 μM for each substrate, the rate of catalysis for K280A was 4 orders of magnitude slower than for wt DMAPP-tRNA transferase.

(6) Mutations Affecting k_{cat}, K_m^{RNA}, and K_m^{DMAPP}. Four mutations generated changes in K_m and k_{cat}, which although not nearly as dramatic as those described above, have significant cumulative effects on the catalytic efficiency of DMAPP-tRNA transferase. The T54A, T108A, and D164A enzymes had 4−12-fold higher values of K_m for both substrates, and values for k_{cat} were reduced by 30−70%. K_m for the Q282A mutant was larger than for the wt, but k_{cat} was slightly higher.

(7) Amino Acids That Interact with A38 in the RNA Substrate. The A36-A37-A38 motif in the anticodon loop of the tRNA substrates is an important structural element for binding (*17*). When A38 is replaced with G in the 17-base RNA stem−loop substrate (Figure 1), K_m^{RNA} increases 6-fold and k_{cat} decreases 7-fold for a combined 40-fold reduction in catalytic efficiency. Since it is likely that A38 interacts with active site residues in the enzyme−substrate complex, replacement of those amino acids with alanine might produce an enzyme that is less sensitive to the A38G change than wt DMAPP-tRNA transferase. This assumption was explored for all of the mutants where K_m^{RNA} or k_{cat} was significantly altered. As reported for wt DMAPP-tRNA transferase, most of the mutants were substantially less active when incubated with A38G RNA instead of the normal substrate under standard conditions of 50 μM RNA and 50 μM DMAPP. However, the activity of the R167A mutant was only 50% lower, and the activity of the K280A enzyme was 2-fold higher. A more detailed analysis showed that for R167A, the A38G substitution lowered K_m^{RNA} 2-fold with a corresponding 8-fold decrease in k_{cat}. We were not able to achieve saturating RNA concentrations with K280A. As seen for the normal RNA substrate, the rate varied linearly with the concentration of the A38G variant up to 150 μM. However, rates were 2-fold higher for the A38G substrate at all RNA concentrations. These results suggest that R167 and K280 are involved in important base-specific contacts with A38 in the RNA substrate.

CD Spectra of wt and Mutant DMAPP-tRNA Transferases. The CD spectrum of wild-type DMAPP-tRNA transferase has a negative maximum molar ellipticity per residue of -11830 deg cm^2 dmol^{-1} at 222 nm, characteristic of proteins containing α-helices. We obtained CD spectra for the mutants with substantially altered kinetic constants to see if the changes were associated with perturbations of their secondary structures. The CD spectra for the T19A, K23A, T24A, D42A, Y47S, K56A, R167A, R170A, R217A, E229A, Q253A, and K280A proteins all had negative maxima at 222 nm and molar ellipticity per residue values between -11470 and -13724 deg cm^2 dmol^{-1}.

DISCUSSION

Prenyltransferases constitute a large family of enzymes that catalyze the electrophilic alkylation of electron-rich acceptors by the hydrocarbon moiety of allylic isoprenoid diphosphates. DMAPP-tRNA transferase belongs to a subcategory of these enzymes where the acceptor, the amino moiety of A37, is a nonisoprenoid molecule. Other prenyltransferases that alkylate nonisoprenoid acceptors include the protein prenyltransferases (sulfhydryl moieties in cysteine residues) (*39*) and geranylgeranylglyceryl phosphate synthase (the C2 hydroxyl group in glyceryl phosphate) (*32*). Except for the protein prenyltransferases (*25*, *40*), virtually no structural information is available for the enzymes that alkylate nonisoprenoid acceptors.

DMAPP-tRNA transferase appears to be one of the most widely distributed enzymes in the nonisoprenoid acceptor group. tRNAs with i^{6}A modifications are found in eubacterial and eukaryotic organisms, and a substantial number of putative ORFs have been identified for DMAPP-tRNA transferase. Although the chemical mechanism for the i^{6}A modification reaction is thought to be an electrophilic alkylation similar to those catalyzed by other prenyltransferases (*41*−*43*), alignments with amino acid sequences for the DMAPP-tRNA transferases do not show any substantial similarity with members of the larger family. The enzyme DMAPP-AMP transferase catalyzes the addition of a dimethylallyl group to the exocyclic amine of AMP during the biosynthesis of plant cytokinins. DMAPP-tRNA transferase and DMAPP-AMP transferase both contain highly conserved stretches of amino acid sequence that resemble the known NTP-binding P-loop motif; however, outside of this region they show little similarity. It is likely, therefore, that the tRNA and AMP transferases represent different classes within the family of prenyltransferases with nonisoprenoid acceptor substrates.

Motifs for binding the isoprenoid diphosphate substrate are prominent in many prenyltransferases. An aspartate-rich DDxxD diphosphate binding motif is found in all of the prenyltransferases that synthesize polyisoprenoid chains with E-double bonds (*28*, *31*, *44*) and many of the terpene cyclases that catalyze a mechanistically similar reaction (*33*−*35*). This motif is thought to play a critical role in binding the substrate and activating the leaving diphosphate group by binding the Mg^{2+} salts of the substrates (*30*, *31*, *44*). The protein prenyltransferases do not contain an analogous aspartate-rich motif. Instead, the nonbridging diphosphate oxygens appear to interact directly with active site lysine, arginine, histidine, and tyrosine residues in an X-ray structure with FPP in the active site (*25*).

Like many other prenyltransferases, DMAPP-tRNA transferase binds its substrates by an ordered mechanism. Pre-steady-state and steady-state kinetic studies both indicate that the tRNA binds before DMAPP (*14*, *16*). K_D for tRNA^{Phe}, measured by fluorescence titration methods, was approximately 20-fold lower than K_m determined from kinetic measurements. In contrast, DMAPP binding was not detected when RNA was absent. However, in the presence of an unreactive RNA minihelix analogue where A37 was replaced with inosine, $K_D^{DMAPP} = 3.4$ μM, essentially the same as K_m for the substrate (*14*). Thus, the anticodon stem−loop region of the tRNA substrate is a required structural element for DMAPP binding by directly interacting with the allylic diphosphate, by causing a conformation change in the enzyme that creates a DMAPP binding site, or both. There is no conserved aspartate-rich motif in the 28 known sequences for DMAPP-tRNA transferase corresponding to the DDXXD sequence seen in many other prenyltransferases and terpene cyclases. The three negatively charged conserved residues (D42, D153, and E229) found in DMAPP-tRNA transferase appear to have different roles. Changing D153 to alanine did not appreciably alter the kinetic constants, while a D42A mutation only produced a modest 20-fold reduction in k_{cat}. In comparison, D → A mutations of conserved aspartates in farnesyl diphosphate synthase resulted in 5−7 order of magnitude decreases in k_{cat} (*30*). The conserved E229 may play a role in RNA binding, as suggested by the substantial increase in K_m^{RNA} for the mutant E229A.

It appears likely that the contacts between DMAPP-tRNA transferase and the diphosphate moiety of DMAPP are more similar to those in protein farnesyltransferase than those in farnesyl diphosphate synthase. Although the DMAPP-tRNA

transferase requires Mg^{2+} for catalysis (*14*), it seems unlikely that the metal ion forms salt bridges between essential carboxylate residues in the active site and oxygens in DMAPP. A more plausible scenario is that the phosphate oxygens make direct contact with positively charged side chains as seen for protein farnesyltransferase. H67F and R217A mutations in DMAPP-tRNA transferase caused large increases in K_m^{DMAPP} without accompanying increases in K_m^{RNA}. The G17-xxxx-G22-K23-T24 element in the enzyme closely resembles the well-characterized P-loop phosphate-binding motif found in a wide variety of NTP-binding proteins (*18*). K23A and T24A mutations resulted in a dramatic increase in K_m^{DMAPP}. It is possible that the P-loop motif sequence binds the nonbridging oxygens in DMAPP and activates the leaving pyrophosphate group. The large increases in K_m^{RNA} for these mutants may also indicate the presence of important contacts between the K23-T24 fragment and the tRNA substrate.

DMAPP-tRNA transferase presumably has a hydrophobic pocket to accommodate the isoprenoid group in DMAPP. The active sites in protein farnesyltransferase and farnesyl diphosphate synthase contain aromatic residues that interact directly with the isoprenoid chain of their respective allylic substrates (*25*, *31*, *44*). DMAPP-tRNA transferase has four completely conserved aromatic residues (Y47, F84, Y111, and W285). However, Y → S, F → A, and W → A mutations did not substantially alter K_m^{DMAPP}. Thus, it is unlikely that these residues interact with the isoprene moiety in DMAPP.

The only aromatic mutant with altered kinetic behavior was Y47S, for which k_{cat} was reduced 100-fold. Interestingly, k_{cat} for the Y47F mutant was similar to that of the wild-type enzyme, indicating that it is the aromatic character of the side chain that is important. Perhaps the aromatic ring in Y47 stabilizes developing positive charge in the electrophilic dimethylallyl group by a "π−cation interaction" during the alkylation reaction (*45*). Similar interactions have been invoked for terpene cyclases as a mechanism for stabilizing electrophilic allylic carbocations in a hydrophobic environment (*33*−*35*).

Since an RNA minihelix with only the anticodon stem−loop sequence for $tRNA^{Phe}$ is an excellent alternate substrate, it is clear that the specific, critical contacts with the DMAPP transferase involve this region. tRNA-guanine transglycosylase (TGT), responsible for biosynthesis of the modified base queuine at position 34 of tRNA, also modifies a stem−loop RNA minihelix (*46*). In the crystal structure of TGT, a conserved RxxR motif was implicated in binding phosphate oxygens in the helical stem of the tRNA anticodon stem−loop minihelix (*23*). R167 and R170 could constitute a similar binding motif in DMAPP-tRNA transferase. The R167A and R170A mutants had greatly elevated values of K_m for both RNA and DMAPP. Finally, R167 and K280 appear to interact with A38 in $tRNA^{Phe}$. Replacement of either amino acid with alanine in DMAPP-tRNA transferase significantly decreases the sensitivity of the enzyme to an A37 → G substitution in the RNA substrate.

In summary, site-directed mutagenesis of conserved amino acids in *E. coli* DMAPP-tRNA transferase implicates several ionic, polar, and aromatic residues in catalysis. Possible roles are DMAPP binding (K23, T24, H67, and R217), tRNA binding (K56, R167, R170, and K280), and stabilization of developing positive charge in the transition state for electrophilic alkylation (T19 and Y47).

REFERENCES

1. Björk, G. R. (1995) in *tRNA: Structure, Biosynthesis and Function*, pp 165−205, ASM Press, Washington, DC.
2. Björk, G. R. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., Ed.) 2nd ed., Vol. 1, pp 861−886, ASM Press, Washington, DC.
3. Buck, M., and Griffiths, E. (1982) *Nucleic Acids Res. 10*, 2609−2624.
4. Gefter, M. L., and Russell, R. L. (1969) *J. Mol. Biol. 39*, 145−157.
5. Eisenberg, S. P., Yarus, M., and Söll, L. (1979) *J. Mol. Biol. 135*, 111−126.
6. Laten, H., Gorman, J., and Bock, R. M. (1978) *Nucleic Acids Res. 5*, 4329−4342.
7. Vacher, J., Grosjean, H., Houssier, C., and Buckingham, R. H. (1984) *J. Mol. Biol. 177*, 329−342.
8. Wilson, R. K., and Roe, B. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 409−413.
9. Blum, P. H. (1988) *J. Bacteriol. 170*, 5125−5133.
10. Gray, J., Wang, J., and Gelvin, S. B. (1992) *J. Bacteriol. 174*, 1086−1098.
11. Buck, M., and Griffiths, E. (1981) *Nucleic Acids Res. 9*, 401−414.
12. Persson, B. C., Ólafsson, Ó., Lundgren, H. K., Hederstedt, L., and Björk, G. R. (1998) *J. Bacteriol. 180*, 3144−3151.
13. Connolly, D., and Winkler, M. E. (1991) *J. Bacteriol. 173*, 1711−1721.
14. Moore, J. A., and Poulter, C. D. (1997) *Biochemistry 36*, 604−614.
15. Leung, H. E., Chen, Y., and Winkler, M. (1997) *J. Biol. Chem. 272*, 13073−13083.
16. Moore, J. A., Mathis, J. R., and Poulter, C. D. (2000) *Biochim. Biophys. Acta 1479*, 166−174.
17. Soderberg, T., and Poulter, C. D. (2000) *Biochemistry 39*, 6546−6553.
18. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) *EMBO J. 1*, 945−951.
19. Milburn, M. V., Tong, L., de Vos, A. M., Brünger, A., Yamaizumi, Z., Nishimura, S., and Kim, S. H. (1990) *Science 247*, 939−945.
20. Berchtold, H., Reshetnikova, L., Reiser, C. O. A., Schirmer, N. K., Sprinzi, M., and Hilgenfeld, R. (1993) *Nature 365*, 124−132.
21. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature 370*, 621−628.
22. Blackwell, J. R., and Horgan, R. (1993) *Phytochemistry 34*, 1477−1481.
23. Romier, C., Reuter, K., Suck, D., and Ficner, R. (1996) *EMBO J. 15*, 2850−2857.
24. Dolence, J. M., Rozema, D. B., and Poulter, C. D. (1997) *Biochemistry 36*, 9246−9252.
25. Strickland, C. L., Windsor, W. T., Syto, R., Wang, L., Bond, R., Wu, Z., Schwartz, H. V., Beese, L. S., and Weber, P. C. (1998) *Biochemistry 37*, 16601−16611.
26. Gebler, J. C., Woodside, A. B., and Poulter, C. D. (1992) *J. Am. Chem. Soc. 114*, 7354−7360.
27. Tsai, H. F., Wang, H., Gebler, J. C., Poulter, C. D., and Schardl, C. L. (1995) *Biochem. Biophys. Res. Commun. 216*, 119−125.
28. Ashby, M. N., and Edwards, P. A. (1990) *J. Biol. Chem. 265*, 13157−13164.
29. Marrero, P. F., Poulter, C. D., and Edwards, P. A. (1992) *J. Biol. Chem. 267*, 21873−21878.
30. Song, L., and Poulter, C. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3044−3048.
31. Tarshis, L. C., Yan, M., Poulter, C. D., and Sacchettini, J. C. (1994) *Biochemistry 33*, 10871−10877.
32. Chen, A., and Poulter, C. D. (1993) *J. Biol. Chem. 268*, 11002−11007.
33. Lesburg, C. A., Zhai, G., Cane, D. E., and Christianson, D. W. (1997) *Science 277*, 1820−1824.

34. Starks, C. M., Back, K., Chappell, J., and Noel, J. P. (1997) *Science 277*, 1815−1820.
35. Wendt, K. U., Poralla, K., and Schulz, G. E. (1997) *Science 277*, 1811−1815.
36. Davisson, V. J., Woodside, A. B., Neal, T. R., Stremler, K. E., Muehlbacher, M., and Poulter, C. D. (1986) *J. Org. Chem. 51*, 4768−4779.
37. Reed, B. C., and Rilling, H. C. (1976) *Biochemistry 15*, 3739−3745.
38. Leatherbarrow, R. J. (1992) *Grafit*, version 3.01, Erithicus Software Ltd., Staines, U.K.
39. Harris, C., and Poulter, C. D. (2000) *Nat. Prod. Rep. 17*, 137−144.
40. Park, H., Boduluri, S., Moomaw, J., Casey, P., and Beese, L. (1997) *Science 275*, 1800−1804.
41. Davisson, V. J., Neal, T. R., and Poulter, C. D. (1993) *J. Am. Chem. Soc. 115*, 1235−1245.
42. Davisson, V. J., and Poulter, C. D. (1993) *J. Am. Chem. Soc. 115*, 1245−1260.
43. Dolence, J. M., and Poulter, C. D. (1999) in *Comprehensive Natural Products Chemistry*, Vol. 5, pp 315−342, Elsevier, Amsterdam.
44. Tarshis, L. C., Proteau, P. J., Kellogg, B. A., Sacchettini, J. C., and Poulter, C. D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 15018−15023.
45. Dougherty, D. A. (1996) *Science 271*, 163−168.
46. Curnow, A. W., and Garcia, G. A. (1995) *J. Biol. Chem. 270*, 17264−17267.

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