Functional Importance of Motif I of Pseudouridine Synthases: Mutagenesis of Aligned Lysine and Proline Residues†

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ABSTRACT: On the basis of sequence alignments, the pseudouridine synthases were grouped into four families that share no statistically significant global sequence similarity, though some common sequence motifs were discovered [Koonin, E. V. (1996) *Nucleic Acids. Res. 24*, 2411−2415; Gustafsson, C., Reid, R., Greene, P. J., and Santi, D. V. (1996) *Nucleic Acids Res. 24*, 3756−3762]. We have investigated the functional significance of these alignments by substituting the nearly invariant lysine and proline residues in Motif I of RluA and TruB, pseudouridine synthases belonging to different families. Contrary to our expectations, the altered enzymes display only very mild kinetic impairment. Substitution of the aligned lysine and proline residues does, however, reduce structural stability, consistent with a temperature sensitive phenotype that results from substitution of the cognate proline residue in Cbf5p, a yeast homologue of TruB [Zerbarjadian, Y., King, T., Fournier, M. J., Clarke, L., and Carbon, J. (1999) *Mol. Cell. Biol. 19*, 7461−7472]. Together, our data support a functional role for Motif I, as predicted by sequence alignments, though the effect of substituting the highly conserved residues was milder than we anticipated. By extrapolation, our findings also support the assignment of pseudouridine synthase function to certain physiologically important eukaryotic proteins that contain Motif I, including the human protein dyskerin, alteration of which leads to the disease dyskeratosis congenita.

The pseudouridine synthases (Ψ synthases)[1] catalyze the isomerization of uridine in RNA to its C-glycoside isomer, Ψ (Figure 1). This isomerization is the most prevalent posttranscriptional modification of RNA, and Ψ has been found in all organisms, occurring in tRNA, rRNA, snRNA, and snoRNA (*1*). The groups of Penhoet (*2*) and Ofengand (*3*−*5*) cloned four genes encoding Ψ synthases, all from *Escherichia coli*: *truA*[2] (*2*), *rluA* (*4*), *truB* (*3*), and *rsuA* (*5*). Analysis of the protein sequences of these four Ψ synthases revealed that they share no statistically significant global sequence similarity. Each enzyme, however, had clear homologues in the database, which led to the grouping of Ψ synthases into four families, each represented by one of the first four cloned Ψ synthase genes (*6*, *7*). Further scrutiny revealed that one short motif (Motif II) occurs in all four families, that another short motif (Motif I) occurs in three families, and that a third short motif occurs in the families represented by RluA and RsuA (*6*). Sequence analysis has also revealed similarity between TruB and physiologically important proteins in yeast (*8*, *9*), *Drosophila melanogaster* (*10*, *11*), rat (*12*), and humans (*13*), and these proteins have been deduced to be Ψ synthases, largely on the basis of the sequence comparisons.

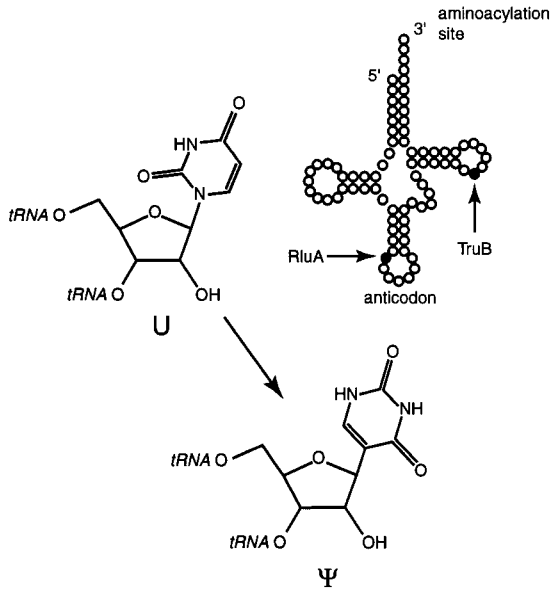

FIGURE 1: Reaction catalyzed by Ψ synthases and the location of uridine residues in *E. coli* $tRNA^{Phe}$ that are isomerized by TruB (U55) and RluA (U32).

Many more Ψ synthases have now been cloned from *E. coli* (*14*−*16*), *Bacillus subtilis* (*17*), yeast (*18*−*20*), mouse (*21*), and human (*21*), but the functional importance of only one residue in the two sequence motifs has been thoroughly investigated. A single aspartic acid residue present in Motif II of all of the Ψ synthases (*22*) has been shown to be catalytically essential in representatives of all four Ψ synthase families (*22*−*25*), likely serving as a nucleophile (*26*). In Motif I, two aligned residues, a proline and a lysine, are

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[1] Abbreviations: Ψ synthases, pseudouridine synthases; Ψ, pseudouridine; tRNA, transfer RNA; T_m, melting temperature.

[2] The gene *truA* was originally named *hisT* and encodes the Ψ synthase that isomerizes uridine residues to the 3′-side of the anticodon of tRNA; we will refer to the encoded enzyme as TruA, but it is also called Ψ synthase I.

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family	Motif I sequence	occurrence lysine	occurrence proline
TruB	^{14}V L L L D K P Q G M S S N D	47/47	44/47
RluA	^{23}I M V V N K P S G L L S V P	81/81	75/81
RsuA	^{62}Y F M L N K P Q G Y V C S T	36/36	34/36

FIGURE 2: Motif I of Ψ synthases (*6*), showing RsuA, RluA, and TruB as representatives of their families. The numbers at the beginning of each sequence denote the position of the first residue. The boxed residues are those mutated in RluA and TruB in the current study, and the frequency of their occurrence in members of the families is indicated to the right. Family members were determined by a single iteration of PSI−BLAST (*34*) with an E-value threshold of 0.001; duplicates and sequences lacking the catalytically essential aspartic acid residue in Motif II were excluded from the statistics.

present in a large majority of members of the families represented by RluA, RsuA, and TruB (Figure 2); the family represented by TruA lacks a discernible Motif I. This work, the first in vitro examination of the functional importance of Motif I, presents the effects of substituting the aligned lysine and proline residues in TruB and RluA, which isomerize, respectively, U55 in tRNA and both U32 in tRNA and U746 in 23S rRNA (Figure 1).

MATERIALS AND METHODS

General. Competent BLR(DE3) pLysS *E. coli* were purchased from Novagen (Madison, WI). Plasmids based on pET15b (Novagen) containing the genes *truB* (*3*) and *rluA* (*4*) were generously provided by J. Ofengand. Since the reports of these plasmids do not assign them names, they will be referred to as pΨ55 (containing *truB*) and pΨ746 (containing *rluA*). Oligonucleotides (OPC-purified grade) were purchased from The Great American Gene Co. (Ramona, CA). QuikChange site-directed mutagenesis kits were purchased from Stratagene (La Jolla, CA), and a Robocycler Gradient 96 Thermocycler (Stratagene) was used for the PCR component of the site-directed mutagenesis. DNA sequencing was performed at the University of Delaware Cell Biology Core Facility using a Long Readir 4200 DNA sequencer (Li-Cor, Inc., Lincoln, NE). HEPES and Tris were purchased from Roche Molecular Biochemicals (Indianapolis, IN). [5-^{3}H]UTP was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Prime RNase inhibitor was purchased from Eppendorf-5′ (Boulder, CO), and Norit SA-3 activated charcoal was purchased from Aldrich Chemical Co., Inc. (Milwaukee). Protein assay kits were purchased from Bio-Rad Laboratories (Hercules, CA). Ni−NTA superflow resin was purchased from QIAGEN (Chatsworth, CA). Spectra/Por dialysis tubing (12000−14000 NMWCO) and all other chemicals were purchased from Fisher Scientific or its Acros Organics division (both in Pittsburgh, PA).

Site Directed Mutagenesis. Mutants of RluA and TruB were generated using the QuikChange site-directed mutagenesis protocol (Stratagene) as previously described (*24*). For the altered RluA, pΨ746 was the parent plasmid, and for the altered TruB, pΨ55 was the parent plasmid. Lysine residues (Lys-19 in TruB and Lys-28 in RluA) were changed to both arginine and methionine, and proline residues (Pro-20 in TruB and Pro-29 in RluA) were changed to both glycine and leucine. All enzymes described here are fused to a His_6·Tag, but references to amino acid positions are in terms of the primary sequence of the native protein, not the His_6·Tagged variant (this particular His_6·Tag adds 20 amino acids to the N-terminus). Mutations were confirmed by sequencing the entire gene. The sequences of all plasmids described in this paper can be found at http://www.udel.edu/chem/mueller. The mutagenesis primers and the names of the resulting plasmids are contained in Table 1.

Overexpression, Purification, and Activity Assay of the Ψ Synthases. Overexpression of altered RluA and TruB enzymes has been described in detail elsewhere (*24*). Induction of expression was achieved by addition of IPTG to a culture (250 mL) of BLR(DE3) pLysS harboring the appropriate plasmid. The cells were harvested 3 h after induction, and the Ψ synthases were purified by chromatography over Ni−NTA resin. The Ψ synthases were prepared for storage by dialysis to remove imidazole (the eluent) as described previously (*27*). Protein concentration was determined using the Bio-Rad protein assay. We calculated correction factors for the response of wild-type RluA and TruB in this dye-binding assay relative to the response of the bovine serum albumin standard by comparing protein concentrations determined by the Bio-Rad and Biuret protein assays (*27*). While the response of both RluA and TruB varied significantly from that of bovine serum albumin in the Bio-Rad assay, the response of the altered and wild-type Ψ synthases agreed within 10%, validating our use of wild-type correction factors. Protein purity (>98%) was confirmed by SDS−PAGE analysis with Coomassie Blue staining (data not shown).

The assay for Ψ synthase activity is a modified version of that reported by Nurse et al. (*3*) and measures the amount of tritium released from C5 when [5-^{3}H]uridine in tRNA substrate is isomerized to Ψ; complete procedures have been published elsewhere (*24*). The substrate for the assay of Ψ synthase activity was the in vitro transcript of *E. coli* $tRNA^{Phe}$ containing [5-^{3}H]uridine, which was prepared as described earlier (*24*). All assays were repeated in duplicate or triplicate, and the rate of tritium release versus time varied less than 10% (generally less than 5%), which is within the uncertainty arising from the measurement of time, volume, and concentrations. The variation of the initial reaction velocity with substrate concentration was fit to the Briggs-Haldane equation using Delta Graph 4.0 (SPSS Inc., Chicago). Errors in the final kinetic parameters k_{cat} and K_m were derived by fitting the extreme values (within the uncertainty) of initial velocity and substrate concentration to the Briggs-Haldane equation; for this error analysis, minimal substrate concentration was paired with maximal initial velocity and vice versa.

CD Spectroscopy and the Determination of Melting Temperatures. Far-UV CD spectra were obtained using a JASCO J-710 spectropolarimeter. Enzyme samples for melting temperature (T_m) determinations were prepared by dialysis against 50 mM potassium phosphate buffer, pH 7.5, containing potassium chloride (100 mM). The CD spectra of the wild-type and altered RluA were acquired in the same buffer, but the CD spectra of the wild-type and altered TruB were acquired in buffer with ammonium chloride (100 mM) substituted for potassium chloride. The protein samples were adjusted to a concentration of 100−200 μg/mL (determined by A_{280nm} after centrifugation to remove precipitate) and loaded into a jacketed cylindrical quartz cell (0.1 cm path

Table 1: Primers for Site-Directed Mutagenesis of TruB and RluA and the Plasmids Encoding the Altered Enzymes[a]

mutation	primers	plasmid
	TruB	
K19M	C GGC GTT TTG TTG CTG GAT ATG CCT CAG GGT ATG TCC AGC GCT GGA CAT ACC CTG AGG CAT ATC CAG CAA CAA AAC GCC G	pBH311
K19R	C GGC GTT TTG TTG CTG GAT CGA CCT CAG GGT ATG TCC AGC GCT GGA CAT ACC CTG AGG CCG ATC CAG CAA CAA AAC GCC G	pBH312
P20G	C GTT TTG TTG CTG GAT AAA GGT CAG GGT ATG TCC AGC AAC GAT GC GC ATC GTT GCT GGA CAT ACC CTG ACC TTT ATC CAG CAA CAA AAC G	pBH313
P20L	C GTT TTG TTG CTG GAT AAA CTT CAG GGT ATG TCC AGC GCT GGA CAT ACC CTG AAG TTT ATC CAG CAA CAA AAC G	pBH314
	RluA	
K28M	T ATT ATG GTG GTC AAC ATG CCG AGC GGT TTG TTG TC GA CAA CAA ACC GCT CGG CAT GTT GAC CAC CAT AAT A	pBH211
K28R	C CAT ATT ATG GTG GTC AAC CGG CCG AGC GGT TTG TTG TCA GTG C G CAC TGA CAA CAA ACC GCT CGG CCG GTT GAC CAC CAT AAT ATG G	pBH212
P29G	G GTG GTC AAC AAG GGG AGC GGT TTG TTG TCA GTG CCG CGG CAC TGA CAA CAA ACC GCT CCC CTT GTT GAC CAC C	pBH213
P29L	G GTG GTC AAC AAG CTG AGC GGT TTG TTG TCA GTG C G CAC TGA CAA CAA ACC GCT CAG CTT GTT GAC CAC C	pBH214

[a] The upper primer is broken into codons, and the changed bases are underlined.

length), which was then connected to a water bath. The time required for thermal equilibration was determined by recording spectra at 2, 5, and 10 min after the water bath reached the set temperature (5 °C, 37 °C, and 50 °C); the spectra recorded at 5 and 10 min varied by less than 2%, so a 5 min equilibration time was judged sufficient. For full spectra, three scans were acquired and averaged, and the spectrum of the buffer was subtracted. The mean residue molar ellipticity $[\theta]$ (deg cm^2/dmol) was calculated using the formula $[\theta] = \theta/(10ncl)$, where θ is the ellipticity in millidegrees, n is the number of amino acid residues per monomer, c is the molarity of enzyme in the sample, and l is the path length of the cell in centimeters.

To determine T_m values, the variation of the CD spectrum with temperature was measured. Since both TruB and RluA give a strong negative ellipticity at 221 nm, θ_{221nm} was used as the measure of folded protein and plotted against temperature to obtain melting curves. After thermal equilibration to 5 °C (5 min), a full spectrum (190−250 nm) was recorded. At every subsequent temperature, a narrower spectrum (205−225 nm) was acquired 10 times and averaged. The temperature was raised in increments of 5 °C in the pre- and posttransition phases and in increments of 2 °C in the transition phase. Uncertainty in θ_{221nm} was assessed by acquiring narrow spectra in triplicate in the pre-transition phase (5 °C, 25 °C), the transition phase (37 °C), and the posttransition phase (65 °C); the three measurements of θ_{221nm} varied by less than 3%. To assess the reversibility of denaturation, spectra were recorded as the temperature was lowered in 5 °C increments.

Data from the melting curves were processed according to the method described by Pace et al. (*28*). The value of θ_{221nm} was used to determine K_{eq} for the folding equilibrium at each temperature. Next, the K_{eq} values were fit to the van't Hoff equation, $\ln K_{eq} = (\Delta H/R)(1/T) + \Delta S/R$. For monomeric enzymes, $K_{eq} = 1$ at T_m, so the x-intercept is $1/T_m$. The uncertainty in T_m was calculated in the same manner but using lines of maximal and minimal slope (rather than the best fit line) to describe the pre- and posttransition phases when determining K_{eq} at each temperature.

RESULTS

Generation and Isolation of Altered RluA and TruB. Site-directed mutagenesis proceeded smoothly, and all mutations were confirmed by sequencing the entire gene. Although primers used to generate the K19R TruB contained an inadvertent mismatch in codon 19, the desired change was obtained. All host cells harboring plasmids containing altered enzymes grew normally, and purification of the altered enzymes proceeded regularly except for the K28M and K28R RluA, which formed inclusion bodies (data not shown) and were not isolated. All other altered Ψ synthases were prepared for storage as reported previously (*27*) but were found to be less stable than their wild-type counterparts. The P20G and P20L TruB began precipitating during dialysis, and no activity remained after 24 h. The K19M and K19R TruB also precipitated, although at a slower rate than the TruB with Pro-20 altered. The stability of the P29G and P29L RluA was comparable to that of the K19M and K19R TruB.

CD Spectra and Melting Temperatures of Altered RluA and TruB. Due to the observed instability of the altered enzymes, CD spectra (Figure 3) were recorded with minimal delay after changing the protein sample into phosphate buffer. All of the altered enzymes differed from their wild-type progenitors, including the saliently reduced intensities of the altered TruB. The T_m was determined (Figure 4, Table 3) for the wild-type and each altered RluA and TruB. For both TruB and RluA, substitution of the aligned proline reduced the T_m with a smaller decrease upon substitution with glycine than with leucine. Thermal denaturation was found to be irreversible for the altered enzymes (no change in θ_{221nm} when the solutions were cooled from 65 °C) and only partially reversible for wild-type TruB and RluA (16% and 18%, respectively, of the initial θ_{221nm} was recovered upon cooling). Such irreversible thermal denaturation is not uncommon (*28*), and in no case was a precipitate observed in the cuvette.

Kinetic Characterization of Altered RluA and TruB. Because of the noted instability of the altered RluA and TruB, all assays were performed within a few hours of the completion of dialysis. The variation of rate with substrate concentration was determined for each purified altered Ψ

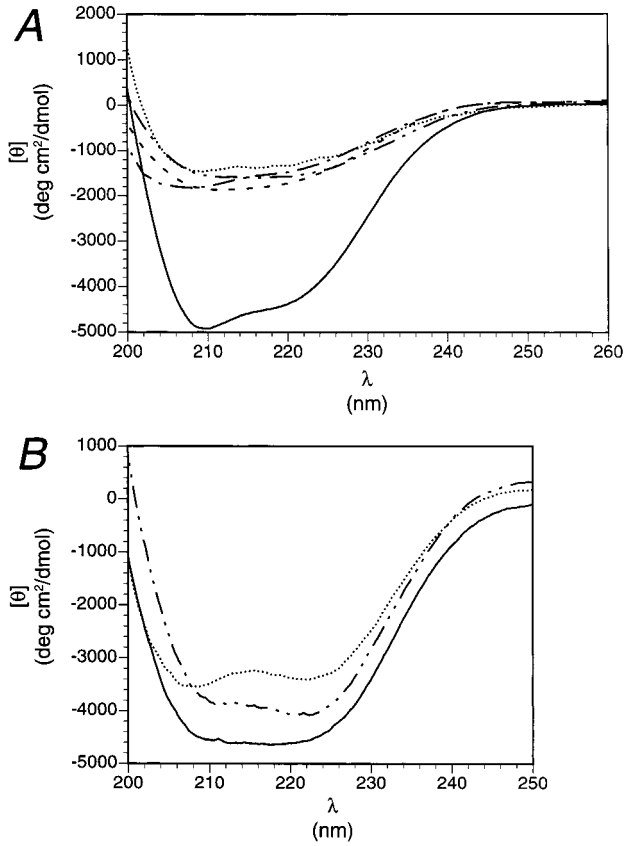

FIGURE 3: CD spectra (A) TruB variants (25 °C). (—) Wild-type; (- - -) K19M; (— — - - —) K19R; (•••) P20G; (— - - —) P20L. (B) RluA variants (5 °C). (—) Wild-type; (•••) P29G; (— - - —) P29L.

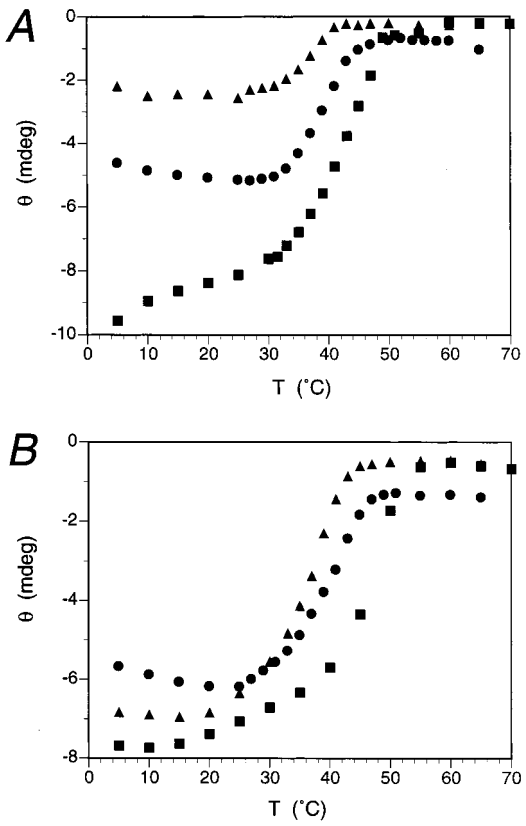

FIGURE 4: Melting curves. (A) TruB, (■) wild-type; (●) P20G; (▲) P20L. (B) RluA, (■) wild-type; (●) P29G; (▲) P29L. All samples were 0.2 mg/mL protein.

synthase (Figure 5), and the data were used to calculate the kinetic parameters k_{cat} and K_m (Table 2). The K19M and K19R TruB both had reduced k_{cat} values, roughly 10-fold for each; K_m values were elevated relative to wild-type TruB, 11-fold for the K19M TruB and 6-fold for K19R TruB. The k_{cat} and K_m values for the P20G TruB were roughly double those of the wild-type enzyme, and the P20L TruB showed 4-fold and 3.5-fold increases in k_{cat} and K_m, respectively, relative to wild-type TruB. Compared to wild-type RluA, both alterations of Pro-29 somewhat reduced k_{cat}, and 3-fold and 4-fold increases in K_m were observed for the P29G and P29L RluA, respectively.

Table 2: Kinetic Parameters for Wild-Type and Altered TruB and RluA

enzyme	k_{cat} (s^{-1})	K_m (nM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
	TruB		
wild-type[a]	0.110 ± 0.008	148 ± 20	7.4×10^5
K19M	0.012 ± 0.0006	1610 ± 160	7.5×10^3
K19R	0.011 ± 0.0006	900 ± 90	1.2×10^4
P20G	0.23 ± 0.01	324 ± 36	7.1×10^5
P20L	0.52 ± 0.03	517 ± 52	1.0×10^6
	RluA		
wild-type[b]	0.099 ± 0.003	108 ± 20	9.2×10^5
P29G	0.061 ± 0.003	318 ± 32	1.9×10^5
P29L	0.051 ± 0.003	455 ± 45	1.1×10^4

[a] The values for wild-type TruB were independently determined and agree well with those that we previously reported (*27*). [b] The data for wild-type RluA are from Ramamurthy et al. (1999) (*27*).

DISCUSSION

Sequence comparisons of the first four cloned Ψ synthases revealed that these enzymes fall into four families with no statistically significant overall similarity (*6*, *7*). All four families, however, share a short sequence motif, Motif II, that contains a catalytically essential aspartic acid residue (*22*−*25*). Another short sequence motif, Motif I (Figure 2), was also identified, though it is not discernible in the family represented by TruA (*6*, *7*).

We have examined the functional importance of Motif I in Ψ synthases of two different families, TruB and RluA. Due to the lack of global sequence similarity between the Ψ synthase families and the very limited number of shared sequence motifs, we reasoned that Motif I would play an important catalytic role. We began our investigation by substituting the highly conserved lysine and proline residues of Motif I (Figure 2). We hypothesized that the lysine would serve as a general acid/base or as an electrophilic catalyst by providing a positive charge, hydrogen bonding, or both, so we substituted the lysine residue with methionine and arginine. The former substitution replaces lysine with a close isostere that cannot function as an electrophilic catalyst, and the latter substitution retains cationic character and the potential for hydrogen bonds, though optimal side-chain orientation might not be achieved in the altered enzyme. Considering the unique conformational properties of the proline residue, we reasoned that it might orient the adjacent lysine residue for its catalytic role. We therefore substituted the proline residue with glycine and leucine, hypothesizing that conformationally permissive glycine might still allow Motif I to adopt a native local structure while more sterically

Table 3: T_m Values for Wild-Type and Altered TruB and RluA

enzyme	T_m (°C)	range[a] (°C)
	TruB	
wild-type	40.2	39.9−41.8
P20G	38.3	38.1−38.8
P20L	35.9	35.3−36.5
	RluA	
wild-type	44.9	44.4−47.8
P29G	37.9	37.3−38.1
P29L	35.0	33.2−35.4

[a] The minimum and maximum T_m derived from the melting curves. The uncertainty was calculated from fitting lines of maximal and minimal slope to the pre- and posttransition phases, which results in asymmetric error. The measured T_m for each of the three variants are significantly different for both RluA and TruB.

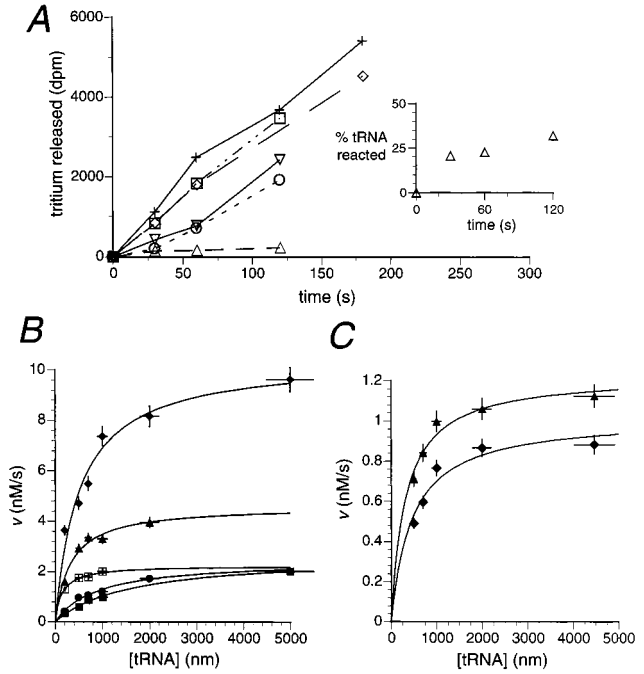

FIGURE 5: (A) Representative plot of tritium release (the measure of Ψ formation) with time catalyzed by the P29L RluA (20 nM). The points are connected for ease in following the data points for each [tRNA]. (△) 270 nM; (○) 500 nM; (▽) 700 nM; (◇) 1000 nM; (□) 2000 nM; (+) 4460 nM. (Inset) Expansion of the data for 270 nM tRNA showing curvature that is not due to substrate depletion (the Ψ synthase reaction is essentially irreversible). (B) Kinetic data for the altered and wild-type TruB. All enzyme concentrations were 20 nM except for the K19M and K19R TruB, which were present at 220 nM. (□) Wild-type TruB; (■) K19M TruB; (●) K19R TruB; (▲) P20G TruB; (◆) P20L TruB. (C) Kinetic data for the altered RluA. Both enzyme concentrations were 20 nM. (▲) P29G RluA; (◆) P29L RluA.

demanding leucine might disrupt the local structure of Motif I.

Site-directed mutagenesis afforded the altered enzymes, and a change in their physical properties was immediately apparent. Substitution of the aligned lysine and proline residues dramatically decreased the stability of RluA and TruB, as indicated by the precipitation of the altered enzymes from solution conditions under which the wild-type enzymes are stable for months. The RluA with Lys-28 changed to either methionine or arginine formed inclusion bodies, indicating precipitation even within the cell. In light of the physical instability of these enzymes, all of the described characterizations were carried out with minimal delay following isolation of the altered TruB and RluA. The CD spectra of the mutant TruB and RluA differ from the wild-type enzymes in the region diagnostic of secondary structure (Figure 3). The sharply reduced intensity of the TruB variants is particularly striking and does *not* arise from precipitation of protein in the sample (protein concentration was determined by A_{280nm} after centrifugation, and no precipitate was observed after the experiments). These observations are consistent with the alteration of Motif I triggering a structural change, which could range from a localized to a widespread disruption of structure. The melting points (Figure 4) of the RluA and TruB with a substituted proline residue were reduced from those of the wild-type enzymes, with the glycine substitution less detrimental to thermal stability than the leucine substitution. These results are in accord with the basic premise of conformational flexibility that led us to choose these particular substitutions.

We next characterized the altered enzymes kinetically (Figure 5, Table 2). Several factors combine to hamper our determination of k_{cat} and K_m. First, both RluA and TruB (even the wild-type enzymes) lose stability under the assay conditions when their concentrations are reduced below 20 nM. Second, the required physical manipulations (quenching of aliquots into acidic suspensions of activated charcoal) preclude accurate time points sooner than 30 s after reaction is initiated. Third, the enzymes bind the tRNA substrate rather tightly: K_m < 200 nM for wild-type TruB and RluA (*27*). Together, these three factors make it exceedingly difficult to acquire data at substrate concentrations that are significantly below the value of K_m while maintaining substrate in excess over enzyme and pseudo-first-order conditions regarding substrate concentration during the measurement of initial velocity. Due to the additional structural instability of the altered RluA and TruB, it is important to note that the plots of tritium release versus time display no curvature over the time frame used to calculate initial velocities (Figure 5, panel A) for all substrate concentrations except the lowest we attempted (270 nM), which was therefore excluded from the analysis. We surmise that the stability (even for the enzymes with a T_m < 37 °C, the assay temperature) arises in part from the presence of tRNA in the assay, for the presence of substrate frequently enhances the structural stability of an enzyme. A reduced rate of precipitation (presumably a higher-order kinetic process) is also expected from the reduced concentration in the assay mixture (20 or 220 nM) compared to the dialyzed sample (∼65 μM RluA and ∼85 μM TruB).

While the kinetic parameters (Table 2) have a higher degree of uncertainty (up to 11% in the values of k_{cat} and K_m) than we would prefer, it is abundantly clear that all of the altered enzymes retain substantial activity. Mindful of the caveats inherent in the interpretation of relatively small changes in values of k_{cat} and K_m, especially in light of the altered structure, we shall now summarize the kinetic ramifications of substituting the aligned Lys and Pro residues in Motif I. Substitution of Lys-19 of TruB with either methionine or arginine reduced k_{cat} by 10-fold and caused similar increases in K_m values. Both the K19M and K19R TruB are, then, 2 orders of magnitude less efficient than wild-type TruB as assessed by k_{cat}/K_m values, with the K19R TruB slightly less impaired. Substitution of Pro-20 of TruB with glycine or leucine leads to nearly parallel increases in k_{cat}

and K_m values, resulting in enzymes that essentially equal wild-type TruB in efficiency. Replacement of Pro-29 in RluA with either glycine or leucine reduced k_{cat} somewhat and produced larger increases in K_m values, leading to enzymes 5-fold and 9-fold less efficient than wild-type RluA. In short, substitution of the lysine residue in Motif I produced noncatastrophic impairment of catalysis, and the proline residue of Motif I could be replaced with even smaller catalytic effect.

All of our data point to a structural rather than a direct catalytic role for the highly aligned lysine and proline residues in Motif I. The in vivo characterization of a yeast homologue of TruB, Cbf5p, is also in accord with the postulated structural role of Motif I (*29*). Cbf5p associates with small nucleolar ribonuclear particles and participates in the maturation of rRNA (*8*, *9*) by catalyzing pseudouridylation (*29*). Carbon and co-workers substituted the aligned proline residue in Motif I of Cbf5p with alanine and examined the phenotypes of yeast that expressed only the altered Cbf5p (*29*). They found that growth was temperature-sensitive but that the level of pseudouridine in rRNA slightly *increased* in cells harboring the Pro→Ala Cbf5p. The investigators concluded that the altered Cbf5p was catalytically active but thermally unstable, causing partial loss of a second function of Cbf5p at normal growth temperatures (*29*). Though the protein context (Cbf5p) in the in vivo study differs from the protein context (TruB) in the in vitro experiments reported here, we are gratified by the consistency of the two sets of observations: the temperature sensitivity of the yeast that rely on the altered Cbf5p agrees well with our measured decrease in melting temperature for the similarly altered TruB, and the high level of pseudouridine in rRNA nicely fits our measured kinetic parameters for the P20G and P20L TruB (greater k_{cat} and similar k_{cat}/K_m values relative to wild-type TruB).

Though our observations do not allow us to draw any but the most general structural conclusions, we propose that the structural changes arise within or are propagated to the active site. Since K_m likely reflects substrate binding for these relatively slow enzymes, the elevated K_m values in the altered RluA and TruB support an altered active site. A structural change at the active site can also accommodate the variation in k_{cat} values. The 10-fold reduction in k_{cat} for the K19M and K19R TruB could easily arise from a perturbed alignment of the substrate tRNA in the active site rather than by direct catalytic participation of Lys-19, in which case a more dramatic catalytic impairment might be anticipated upon substitution of the residue.

The crystal structure of the Ψ synthase TruA has recently been published (*30*) and might be expected to illuminate the nature of Motif I. However, TruA has neither a discernible Motif I nor statistically significant overall sequence similarity to either RluA or TruB (*6*, *7*). The report of the crystal structure also points out that the mode of tRNA binding is likely to differ between TruA and TruB (*30*), so extrapolation of the TruA structure to the two enzymes that we examined should be made with extreme caution. In light of these caveats, we examined the region of TruA where a Motif I would lie based on the relative positioning of Motifs I and II in the three families that contain both. *E. coli* TruA contains two lysine residues in this region, Lys-22 and Lys-38, but they are neither well-conserved nor found next to proline residues. Both Lys-22 and Lys-38 are found in secondary structure elements that border the tRNA binding cleft: the former in a flexible loop, the latter in an α-helix that extends away from the active site (*30*). While these observations are consistent with our interpretation that substitution of Motif I residues triggers a structural change that impairs substrate binding and alignment, we must await the insights to be afforded by the structure of a Ψ synthase from a family (or perhaps even from each family) other than that of TruA before we can confidently assign a role to Motif I.[3]

In the absence of such a structure, we must rely on indirect evidence to elucidate amino acid residues critical for function. Sequence alignments provide a powerful tool in this regard and were used to identify an aspartic acid residue in motif II (*22*) that is absolutely critical for activity in Ψ synthases of each family (*22*−*25*). The experiments described here were undertaken to investigate the role of the lysine and proline residues in Motif I (Figure 2) that are highly conserved within three families of Ψ synthases (*6*, *7*). The proline residue in Motif I is very highly conserved among the Ψ synthase families that bear a Motif I, and the adjacent lysine residue is completely conserved, which led us to predict that the lysine residue participates directly and critically in catalysis. Instead, our findings suggest that this lysine residue plays a structural rather than a direct catalytic role in RluA and TruB. The relatively subtle effects of substituting the highly conserved lysine residue serves as a reminder of the limitations of sequence data in the elucidation of enzyme function: sequence analysis is remarkably powerful, but the roles of implicated amino acid residues must be verified experimentally. In this instance, the prediction of a functional role for the aligned residues in Motif I was borne out, but in an unanticipated manner, highlighting the need for experimental elucidation of functional "leads" derived from genomic databases.[4]

Finally, we address the bearing of our findings on the proposal that some physiologically critical eukaryotic enzymes are Ψ synthases. This proposal rests in part on sequence alignments that reveal strong similarity to TruB, notably the presence of motifs I and II, in domains of Cbf5p in yeast (*8*, *9*), MFL in *D. melanogaster*[5] (*10*, *11*), and NAP57 in rat (*12*). These three proteins are orthologues that associate with small nucleolar ribonuclear particles and play roles in the maturation of rRNA. It has recently been shown that mutation of the aspartic acid residue in Motif II of Cbf5p abolishes rRNA pseudouridylation (*29*). As discussed above, substitution of the proline residue of Motif I in Cbf5p leads to a temperature-sensitive phenotype (*29*) that is consistent with our observation that structural instability arises from substitution at this position.

The human orthologue of Cbf5p, MFL, and NAP57 is dyskerin, the alteration of which leads to the disease dyskeratosis congenita (*13*), and it was proposed that

[3] Crystals have been grown of a catalytically active fragment of the *E. coli* Ψ synthase RluD, a member of the RluA family (*33*).

[4] We harbor no delusions of expertise in the analysis of sequence data, but we do fancy ourselves to be as savvy as "typical" enzymologists in such analysis.

[5] The homologue from *D. melanogaster* is also known as Nop60B; the gene encoding the protein is alternately known as *Nop60B* and *minifly* (*mfl*).

defective rRNA processing might account for the disease (*13*, *31*). Recently, dyskerin has been reported to be associated with telomerase, which maintains telomeres at chromosomal ends; cell lines with defective dyskerin were found to have reduced levels of telomerase activity but apparently normal rRNA biogenesis (*32*). The phenotype of dyskeratosis congenita was concluded to arise not from defective rRNA processing but rather from a lack of telomerase activity and resulting chromosomal instability in affected tissues (*32*). Because we did not examine dyskerin itself, our results cannot resolve whether dyskerin is, indeed, a Ψ synthase, let alone distinguish between the alternate hypotheses concerning the cause of the diseased state. However, our finding that Motif I is functionally important in TruB supports the hypothesis that dyskerin and its orthologues are Ψ synthases. Our work also broadens the examination of Motif I to include two *E. coli* enzymes and suggests that the role of Cbf5p in pseudouridylation (*29*) is not peculiar to this one yeast enzyme but quite likely extends to all of the enzymes that contain Motifs I and II.

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