Phenylalanyl-tRNA Synthetase Contains a Dispensable RNA-Binding Domain that Contributes to the Editing of Noncognate Aminoacyl-tRNA†

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Received March 19, 2006; Revised Manuscript Received May 7, 2006

ABSTRACT: Phenylalanyl-tRNA synthetase (PheRS) is a multidomain $(\alpha\beta)_2$ heterotetrameric protein responsible for synthesizing Phe-tRNA^{Phe} during protein synthesis. Previous studies showed that the α subunit forms the catalytic core of the enzyme, while the β subunit contains a number of autonomous structural modules with a wide range of functions including tRNA anticodon binding and editing of the misaminoacylated species Tyr-tRNA^{Phe}. The B2 domain of the β subunit is a structural homologue of the EMAPII/OB fold, which has been shown in other systems to contribute to tRNA binding. Structural studies of PheRS indicated that the B2 domain is distant from bound tRNA^{Phe}, leaving the role of this module in question. On the basis of homology modeling with other EMAPII domain-containing proteins, the 110 amino acid B2 domain was deleted to produce PheRSΔB2. Full-length PheRS and PheRSΔB2 showed comparable kinetics for in vitro aminoacylation, and both enzymes complemented a defect in phenylalanylation in vivo. PheRSΔB2 showed a 2-fold drop compared to full-length PheRS in the catalytic efficiency (k_{cat}/K_M) of Tyr-tRNA^{Phe} hydrolysis, suggesting a role for the B2 domain in post-transfer editing. A comparison of tRNA binding by full-length PheRS and PheRSΔB2 indicated that the B2 domain acts as a secondary tRNA-binding site that could contribute to editing by promoting the translocation of mischarged tRNA to the editing site of PheRS. This proposed role for the B2 domain of PheRS is consistent with previous studies, suggesting that the highly conserved EMAPII fold is able to modulate the affinity of tRNA for its primary binding site.

The aminoacyl-tRNA synthetases (aaRSs)[1] act during the first step of protein biosynthesis by ensuring the correct aminoacylation of a given tRNA with its cognate amino acid. The fidelity and specificity of contemporary aminoacylation partly stems from the evolutionary acquisition by aaRSs of additional structural modules that enhance tRNA selectivity or proofread erroneously synthesized noncognate aminoacyl-tRNAs (Aa-tRNAs) (*1*). The modularity of the aaRS family is particularly well-illustrated by phenylalanyl-tRNA synthetase (PheRS), a heterotetrameric oligomeric $(\alpha\beta)_2$ enzyme with 11 distinct structural domains; A0−A2 in the α subunit and B1−B8 in the β subunit (*2*, *3*). The oligomeric structure and modular organization of PheRS display significant evolutionary variability. The eukaryotic cytosolic and archaeal enzymes lack domains A0 and B2, while the mitochondrial and chloroplast enzymes are monomers consisting only of the α subunit fused to the B8 domain (*4*−*6*). The minimal structure of the mitochondrial enzyme suggests that many of the domains of the heterotetrameric enzyme may be dispensable for canonical phenylalanylation activity. Biochemical evidence (*7*−*9*), corroborated by the crystal structure of *Thermus thermophilus* PheRS in complex with its cognate tRNA (*10*), clearly demonstrated that only four domains of the protein are directly involved in aminoacylation; the α subunit (A0−A2) carries the catalytic site, and the B8 domain specifically interacts with the anticodon of tRNA^{Phe}. The presence of other modules appended to the A0−A2/B8 core of PheRS correlates with additional functions attributed to the protein. The B6−B7 domain is required for dimerization, while the B3−B4 domain harbors an editing site for hydrolysis of the misaminoacylated species Tyr-tRNA^{Phe}, which is synthesized at a low but significant frequency by PheRS (*11*, *12*). The B5 domain contains a helix−turn−helix (HTH) motif found to specifically interact with DNA (*13*), in agreement with the implication of PheRS in gene regulation, suggested from the relation between the overproduction of the human PheRS α subunit and the myeloid leukemia phenotype (*14*).

The only modules of PheRS that remain biochemically uncharacterized are B1 and B2, both of which have strong homology to known nucleic-acid-binding domains. The B1 domain, like B5, contains an HTH motif and is believed to contribute to the DNA-binding activity of PheRS. The B2 domain was first noted to bear a high structural similarity to the anticodon-binding domain of the class IIb aaRSs (*15*), all of which contain the widely distributed oligonucleotide-binding (OB) fold motif. The OB-fold or EMAPII domain,

† This work was supported by Grant MCB-0344002 from the National Science Foundation.

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[1] Abbreviations: Aa-tRNA, aminoacyl-tRNA; aaRS, aminoacyl-tRNA synthetase; PheRS, phenylalanyl-tRNA synthetase.

10.1021/bi060549w CCC: $33.50
Published on Web 07/06/2006

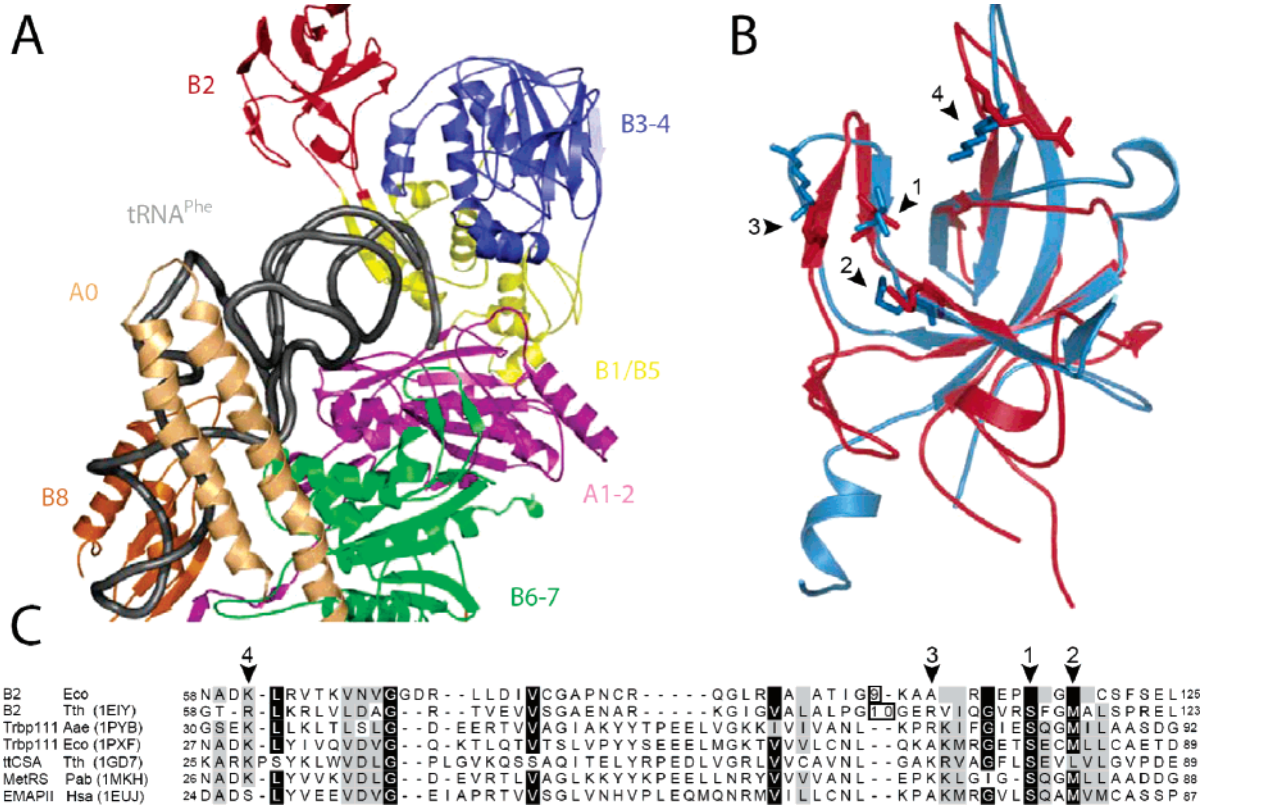

FIGURE 1: Structural analysis of the B2 domain of PheRS. (A) Crystal structure of PheRS in complex with its cognate tRNA. Only one heterodimer ($\alpha\beta$) of the PheRS $(\alpha\beta)_2$ heterotetramer is represented. The tRNA makes specific contacts with domains A1−A2 and with domains A0 and B8 of the second heterodimer (not shown). (B) Superposition of the B2 domain and the protein Trbp111 from *A. aeolicus*. The B2 domain is shown in red, and the protein Trbp111 is shown in blue. (C) Structural alignment of the closest paralogues of the B2 domain. Trbp111 of *E. coli* (Eco) and *A. aeolicus* (Aae), ttCSA of *T. thermophilus* (Tth), the C-terminal domain of the MetRS of *P. abyssi* (Pab), and the EMAPII domain of the p43 protein of *Homo sapiens* (Hsa). Identical and similar amino acids conserved in $^6/_7$ of the sequences are shaded in black and gray, respectively. The four most important positions for tRNA binding identified experimentally in Trbp111 are indicated by arrows (*25*).

which is made of seven coiled β sheets capped by an α helix, is widely distributed among RNA- and DNA-binding proteins including certain cell-signaling molecules (EMAPII and p43) and a variety of free-standing tRNA-binding proteins. In eubacterial methionyl-tRNA synthetases (MetRS), a paralogue of the EMAPII domain is fused to the C terminus of the protein and stimulates the interaction with tRNA (*16*). In eukaryotic MetRSs, the EMAPII domain is found both as a C-terminal appendage and as a free-standing protein (Arc1p), and in both cases, it assists in the recognition of $tRNA^{Met}$ (*17, 18*). The contribution of these domains to tRNA binding by MetRS is limited, however, and recent characterizations of other free-standing tRNA-binding proteins containing EMAPII domains (Trbp111 and ttCsa) suggested a possible additional role in the stabilization of the tRNA structure (*19*−*21*).

Prior to the resolution of the structure of PheRS in complex with its cognate tRNA, the EMAPII-like B2 domain had been considered a likely candidate to participate directly in tRNA recognition. Surprisingly, the structure of the complex did not reveal any direct interactions between B2 and tRNA. Moreover, a recent computational study proposed that the B2 domain might instead be involved in tRNA recruitment to PheRS by long-range electrostatic interactions (*22*). To further investigate the role of the B2 domain, we have now deleted this region of the protein and studied the effects of its removal on the in vitro and in vivo activities of PheRS.

EXPERIMENTAL PROCEDURES

General Procedures. Plasmid-encoding yeast cytosolic $tRNA^{Phe}$ was a gift from O. Uhlenbeck (Northwestern University). The *Escherichia coli* strain NP37 carrying the mutation *pheS*5(ts) was a gift from the Genetic Stock Center (Yale University). *E. coli* XL1-Blue/pQE31-FRSec-αA294G, producing His_6-tagged *E. coli* PheRSαA294G carrying the point mutation A294G in the active site of the enzyme, was a gift from D. A. Tirrell (California Institute of Technology). All His_6-tagged *E. coli* PheRS variants were purified by nickel-affinity chromatography (Qiagen) and stored at −20 °C in 100 mM Tris-HCl buffer (pH7.5), 5 mM β-mercaptoethanol, and 50% glycerol. To delete the B2 domain from the β subunit of *E. coli* PheRS, the vector pQE31-FRSec-αA294G was used as a template for polymerase chain reaction (PCR) amplification with two oligonucleotides (5′-CACTAGTGCTGCCGGCAACCGGTTC-3′ and 5′-CACTAGTGATGACAACACCATCGAAATCAGC-3′) complementary to the flanking region of the B2 domain. The PCR product was digested with *Spe*I and *Dpn*I, purified by agarose gel electrophoresis, and religated into the original vector with T4 DNA ligase. The sequence of the resulting plasmid, deprived of the region coding for the B2 domain, was checked by sequencing, and the resulting plasmid was transformed into *E. coli* XL1-Blue for overproduction of PheRS αA294G$\beta\Delta$B2.

Preparation of in Vitro Transcripts of tRNA. In vitro transcription was conducted as described earlier (*23*). tRNA transcripts were ethanol-precipitated and resuspended by heating in 10 mM Na-Hepes (pH 7.2), 1 mM Na_2EDTA, and 7 M urea, loaded onto a Hiprep 16/10 Q FF column (Amersham Bioscience), and eluted with a 0−1 M NaCl gradient in the loading buffer. The fractions containing tRNA were pooled, ethanol-precipitated, desalted on a PD10 column (Amersham Bioscience) against 10 mM Hepes (pH 7.2), reprecipitated with ethanol, dried, resuspended in 10 mM Hepes (pH 7.2) and 2 mM $MgCl_2$, and finally refolded by incubation for 1 min at 80 °C followed by slow cooling to room temperature.

ATP−PPi Exchange Reaction. Reactions were carried out at 37 °C in 100 mM Na−Hepes (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, 2 mM NaF, 2 mM ATP, 2 mM [^{32}P]PPi (1 cpm/pmol), 2 mM Phe, and 2−8 nM PheRSαA294G or PheRSαA294G$\beta\Delta$B2. Aliquots (25 μL) were removed during the first 5 min of the reaction and added to a solution containing 1% charcoal, 5.6% $HClO_4$, and 75 mM PPi. The charcoal was filtered through a 3MM Whatman filter disk under vacuum and washed 3 times with 5 mL of water and once with 5 mL of ethanol. The filters were dried, and the radiolabeled ATP bound to the charcoal was counted by liquid scintillation counting (Ultima Gold, Packard Instrument Co.).

Aminoacylation Assay. Aminoacylation was performed in 100 mM Na−Hepes (pH 7.2), 30 mM KCl, 2 mM ATP, 10 mM $MgCl_2$, and 50 μM L-[^{3}H]Phe (500 cpm/pmol). PheRS (3 or 40 nM) was used with tRNA^{Phe} transcripts from *E. coli* or yeast, respectively, at concentrations of 1−5 times the K_M. Aliquots (15 μL) were periodically removed and spotted on 3MM filter disks (Whatman), washed 3 times in 10% trichloroacetic acid, and dried. The amount of radioactivity retained was determined by liquid scintillation counting.

Trans-Editing Assay. Tyr-tRNA^{Phe} was prepared with 0.5 μM mitochondrial PheRS A333G as described previously (*5*) in an aminoacylation reaction containing 30 μM [^{3}H]-Tyr (180 cpm/pmol) and 5 μM in vitro transcribed *E. coli* tRNA^{Phe}. Reaction mixtures contained 100 mM Na−Hepes (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, 1 μM [^{3}H]Tyr-tRNA^{Phe}, and 3 nM PheRS. The mixture was incubated at 37 °C, and the trans-editing reaction was followed by measuring the remaining radiolabeled aa-tRNA in aliquots of 15 μL after 0−6 min of incubation as described for the aminoacylation assay (see above).

Cis-Editing Assay. Pre- and post-transfer editing of misactivated Tyr was measured as ATP consumption catalyzed by PheRS in the presence or absence of 2 μM in vitro transcribed active tRNA^{Phe} from *E. coli*. A 15 μL reaction contained 2 mM Tyr, 2 mM [γ-^{32}P]ATP (5 cpm/pmol), 100 mM Hepes-Na (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, and 2 units/mL of yeast inorganic pyrophosphatase (Roche). The reaction was performed at 37 °C and was initiated by the addition of 1 μM PheRS. At variable times, ranging from 0 to 20 min, the reaction was quenched by mixing 2 μL of sample with 2 μL of glacial acetic acid. The remaining [γ-^{32}P]ATP and the [^{32}P]Pi formed during the reaction were separated by thin-layer chromatography (TLC) on a PEI cellulose plate (Sigma). The TLC plate was subsequently developed in 0.7 M potassium phosphate (pH 3.5), and the labeled products were visualized and quantified on a Storm phosphorimager (Amersham Biosciences). The concentration of Pi (in millimolars) formed during the reaction was calculated by multiplying the measured Pi/ATP ratio by the initial concentration of ATP (2 mM).

K_D Determination of PheRS for tRNAs. Transcripts corresponding to *E. coli* or yeast (cytosolic) tRNA^{Phe} were radiolabeled with [α-^{32}P]ATP using nucleotidyl-tRNA transferase as previously described (*5*). Binding-assay media contained 150 nM PheRS (corresponding to 300 nM binding sites) and 0−2.5 μM labeled tRNA transcript in 100 mM Na−Hepes (pH 7.2), 30 mM KCl, and 10 mM $MgCl_2$. After incubation for 10 min at room temperature, 120 μL of binding media was filtered under vacuum onto a 0.45 μm Protran BA85 nitrocellulose membrane (Whatman). Filters were washed twice with 1 mL of 0.5× binding buffer. The filters were then dried, and the radioactivity was determined as described in the aminoacylation assay. The filter retention yields for PheRSαA294G and PheRSαA294G$\beta\Delta$B2, determined in the presence or absence of *E. coli* tRNA^{Phe} transcript, were 60% for both proteins as determined by measuring the amount of unbound protein on the filter with the Bradford assay. In parallel, the active binding sites of the proteins were determined experimentally in the presence of [^{14}C]Phe and ATP (*24*). This revealed that 129 ± 4 and 142 ± 2 nM binding sites of PheRSαA294G and PheRSαA294G$\beta\Delta$B2, respectively, were found active and able to form a titrable complex with [^{14}C]Phe-adenylate. As seen by others (*25*), at low concentrations of tRNA^{Phe}, only one PheRS acceptor site was saturated. The K_D of a second tRNA-binding site was also determined in the presence of high concentrations of transcript (0−10 μM) and was estimated to be 2 μM, representing a 20 times higher value than the K_D for the first binding site (data not shown). The calculation of the dissociation constant (K_D) for the first binding site was then determined with a low-range concentration of tRNA^{Phe} (0−2.5 μM) and by plotting the concentration of bound ligand (B) as a function of the total amount of tRNA^{Phe} added (total ligand, Lt) according to the following equation (*26*):

$$B = \frac{(K_D + Lt + Rt) - \sqrt{(K_D + Lt + Rt)^2 - 4(Lt \times Rt)}}{2}$$

where Rt is the total concentration of PheRS. For each K_D determination, the percent of occupancy was calculated as being the ratio of the amount of complexed [α-^{32}P]tRNA^{Phe} divided by the amount of active sites as determined with the active-site titration assay.

RESULTS

The B2 Domain of PheRS Is a Structural Homologue of Known tRNA-Binding Proteins. The Protein Data Bank was searched for structures similar to the B2 domain of *T. thermophilus* PheRS using VAST (http://www.ncbi.nlm.nih.gov/Structure/VAST/vastsearch.html). While a wide variety of OB-fold-containing proteins were identified that showed a low similarity to PheRS, all of the proteins displaying a root mean square (rms) of less than 1.5 Å and a percentage similarity for aligned residues greater than 20% belonged to the trbp-EMAP-CsaA family. Many of these proteins have

Table 1: Steady-State Kinetic Parameters of the ATP−PPi Exchange Reaction in the Presence of Phe and the Phenylalanylation of $tRNA^{Phe}$ Transcript[a]

PheRS	PheRS αA294G			PheRS αA294Gβ∆B2		
	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} μM^{-1})	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} μM^{-1})
ATP−PPi exchange						
Phe	5.3 ± 0.7	200 ± 30	37	4.9 ± 0.5	196 ± 20	40
$tRNA^{Phe}$ phenylalanylation						
$tRNA^{Phe}$ *E. coli*	2.7 ± 0.1	49 ± 0.1	18	2.5 ± 0.4	47 ± 4	19
$tRNA^{Phe}$ *S. cerevisiae*	2.2 ± 0.02	4.6 ± 1.3	2.1	4.1 ± 0.3	6.7 ± 1	1.6

[a] The values are the average of at least three independent determinations.

previously been shown to bind tRNA, with the best characterized examples being Trbp111 from *Aquifex aeolicus* (1PYB) and *E. coli* (1PXF) (*27*), CsaA from *T. thermophilus* (1GD7) (*20*), the p43 component of the human multisynthetase complex (1FL0) (*28*), and the C-terminal domain of *Pyrococcus abyssi* MetRS (1MKH) (*16*). Guided by the residues previously shown to form the binding interface between Trbp111 of *E. coli* and $tRNA_i^{Met}$, we superimposed the structures of Trbp111 and the B2 domain of *T. thermophilus* PheRS (Figure 1A). In agreement with earlier studies (*28*), the B2 domain cannot dimerize, because the putative interface defined on Trbp111 is hidden by the rest of the β subunit of PheRS. Sequence alignments of the B2 domain with other members of the trbp-EMAP-CsaA family (Figure 1B) revealed that all of the residues reported as being important for tRNA binding by Trbp111 are well-conserved in PheRS, suggesting that the B2 domain might contribute to substrate binding and possibly aminoacylation.

The B2 Domain Is Dispensable for Phenylalanylation in Vitro. To investigate the effect of the B2 domain on phenylalanylation, residues 42−151 of the β subunit of PheRS were replaced by the dipeptide Thr−Ser, which would be expected to form a flexible hydrophilic loop. The deletion was made in the PheRSαA294G background to facilitate the monitoring of both cognate phenylalanylation and the editing of noncognate aminoacylation products (see below) (*11*). Deletion of the B2 domain did not affect the phenylalanine activation step because the kinetic parameters determined for the cognate ATP/PPi exchange reaction did not vary significantly (Table 1). These data suggest that the deletion of B2 did not interfere with the proper folding of the catalytic site, a conclusion supported by active-site titration, which showed a similar percentage of active molecules for both enzymes (see the Experimental Procedures). Steady-state kinetic analyses for aminoacylation of in vitro transcribed *E. coli* $tRNA^{Phe}$ (Table 1) showed that neither the k_{cat} nor the K_M for aminoacylation changed following the deletion of the B2 domain. Some differences were observed in the kinetic parameters for in vitro transcribed yeast cytosolic $tRNA^{Phe}$, most notably a 2-fold increase in K_M upon deletion of the B2 domain.

The B2 Domain Is Dispensable for Phenylalanylation in Vivo. In vitro reconstitution of the phenylalanylation of *E. coli* $tRNA^{Phe}$ suggested that the B2 domain may be dispensable for the canonical activity of PheRS. To investigate if the dispensability of the B2 domain held true in vivo and also to probe whether the B2 domain contributes instead to some other essential cellular function, we attempted to complement the *E. coli* strain NP37, which encodes a temperature-sensitive PheRS variant (*29*). NP37 was transformed with different plasmids expressing the *E. coli* or yeast PheRS-encoding genes and the resulting transformants grown at permissive or restrictive temperature (Figure 2). Production of yeast PheRS, which is unable to efficiently charge *E. coli* $tRNA^{Phe}$ in vitro, was not able to complement the temperature-sensitive allele at the restrictive temperature. In contrast, both *E. coli* PheRSαA294G and PheRSαA294Gβ∆B2 rescued the growth phenotype of NP37 at the restrictive temperature, indicating that the B2 domain is not essential for the survival of *E. coli*.

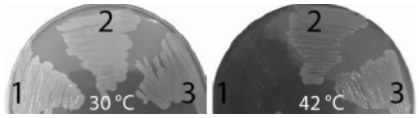

FIGURE 2: Rescue of the thermosensitive growth phenotype of *E. coli* strain NP37. The strain NP37 carrying the thermosensitive mutation *pheS*5(ts) was transformed by an expression vector producing PheRS from yeast (1), PheRSαA294G (2), or PheRSαA 294Gβ∆B2 (3) and plated and incubated for 24 h at permissive (30 °C) or restrictive (42 °C) temperature on LB agar plates containing ampicillin (100 mg/L).

The B2 Domain Does Not Enhance tRNA Folding. It was recently shown that the protein Trbp111 is able to promote the folding and assembly of tRNA (*21*). Because Trbp111 is a close structural homologue of the B2 domain, the ability of full-length and truncated PheRS to promote tRNA folding was investigated. To determine the population of active and correctly folded tRNA, the level of aminoacylation was determined for in vitro transcribed $tRNA^{Phe}$ from *E. coli* in the presence of the PheRSαA294G and PheRSαA294Gβ∆B2. The same level of aminoacylation was obtained with both enzymes, indicating that the B2 domain did not increase the population of active tRNAs (60%). To further investigate the folding activity of PheRS toward tRNA, the mitochondrial $tRNA^{Phe}$ from *Saccharomyces cerevisiae* was also tested. This $tRNA^{Phe}$ transcript is only charged to any significant degree by the corresponding mitochondrial PheRS, albeit only to a level of about 10%, reflecting its propensity to remain mostly in a misfolded state. Numerous other mitochondrial tRNAs have also been reported to have a poor folding capacity in vitro, a property attributed to their unusually high A/U content (*30*). Recent reports indicate that the proportion of correctly folded mitochondrial tRNA transcripts can be enhanced in vitro by the addition of the cognate aaRS during their refolding (*31*), prompting us to test the capacity of PheRS to improve the folding of in vitro transcribed yeast mitochondrial $tRNA^{Phe}$. *E. coli* PheRS enhanced the folding of mitochondrial $tRNA^{Phe}$ by about 50%, despite the inability of the enzyme to aminoacylate this transcript (Figure 3). The ability of PheRS to promote

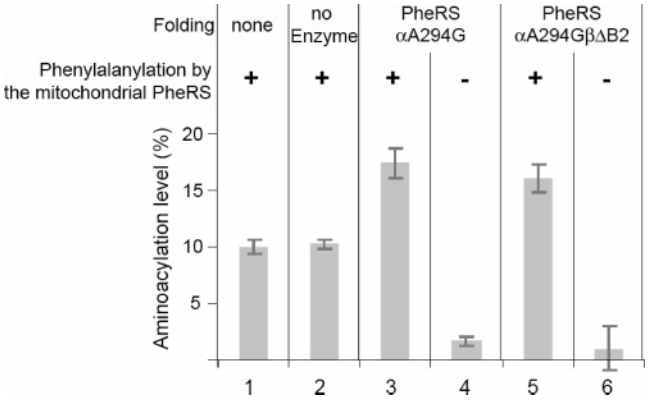

FIGURE 3: Effect of the B2 domain of PheRS on the folding of mitochondrial tRNA^{Phe}. The charging level of the mitochondrial tRNA^{Phe} transcript by the mitochondrial PheRS was 10% (lane 1). The transcript was folded for 10 min at 37 °C in an aminoacylation media in the absence of enzyme (lane 2) or in the presence of 0.1 μM *E. coli* PheRSαA294G (lanes 3 and 4) or PheRSαA294GβΔB2 (lanes 5 and 6). After the folding step, the proportion of folded and active tRNA was measured by phenylalanylation of the transcript by the addition to the folding media of 0.1 μM mitochondrial PheRS, 2 mM ATP, and 30 μM [^{14}C]Phe (lanes 2, 3, and 5). *E. coli* PheRSαA294G and PheRSαA294GβΔB2 were unable to aminoacylate mitochondrial tRNA^{Phe} (lanes 4 and 6).

folding was found to be independent of the B2 domain, because both PheRSαA294G and PheRSαA294GβΔB2 similarly increased the proportion of active mitochondrial tRNA^{Phe}.

The B2 Domain Enhances Editing of Tyr-tRNA^{Phe} by PheRS. In addition to its role in cognate aminoacylation and tRNA folding, PheRS is also known to function in editing by hydrolyzing specifically the misaminoacylated species Tyr-tRNA^{Phe} and not the cognate species Phe-tRNA^{Phe} (*11*, *32*). The editing active site is located in the B3/B4 domain adjacent to B2, raising the possibility that the B2 domain might function as a secondary tRNA-binding site responsible for directing the noncognate Tyr-tRNA^{Phe} toward the editing site. This possibility was addressed by measuring the cis- and trans-editing activities of PheRSαA294G and PheRSαA294GβΔB2 toward Tyr-tRNA^{Phe} (Figure 4). Both enzymes displayed comparable levels of tRNA-dependent or tRNA-independent cis-editing activity, observed as ATP consumption during the futile cycle of misactivation and hydrolysis of the noncognate substrate. However, the trans-editing activity toward preformed Tyr-tRNA^{Phe} was different for full-length and truncated PheRS, with the removal of the B2 domain resulting in a 50% decrease in the initial rate of hydrolysis. The possible role of the B2 domain in promoting editing was supported by steady-state kinetic analysis, which revealed a 2-fold decrease in the k_{cat}/K_M for hydrolysis upon removal of B2 (22 ± 0.6 versus 13 ± 0.5 min^{-1} μM^{-1} for PheRSαA294G and PheRSαA294GβΔB2, respectively).

The B2 Domain of PheRS Promotes Heterologous tRNA Binding. The contribution of the B2 domain to trans editing of mischarged tRNA suggests that it might also contribute to tRNA binding. The propensity of tRNA^{Phe} to form a complex with both full-length and truncated PheRS was examined using filter binding assays on nitrocellulose disks, where fixed amounts of protein were titrated against increasing amounts of 3′-radiolabeled tRNA^{Phe} transcripts from *E. coli* and yeast (cytosolic). Deletion of the B2 domain affected the affinity of both tRNAs for PheRS (Figure 5 and Table 2), increasing by 30% the affinity for *E. coli* tRNA^{Phe} and decreasing the affinity for yeast cytosolic tRNA^{Phe} by 45%. The fraction of occupied binding sites was similar for both enzymes with the *E. coli* substrate but differed significantly for yeast cytosolic tRNA^{Phe}, which showed 50% occupancy of PheRSαA294G and 30% occupancy of PheRSαA294GβΔB2.

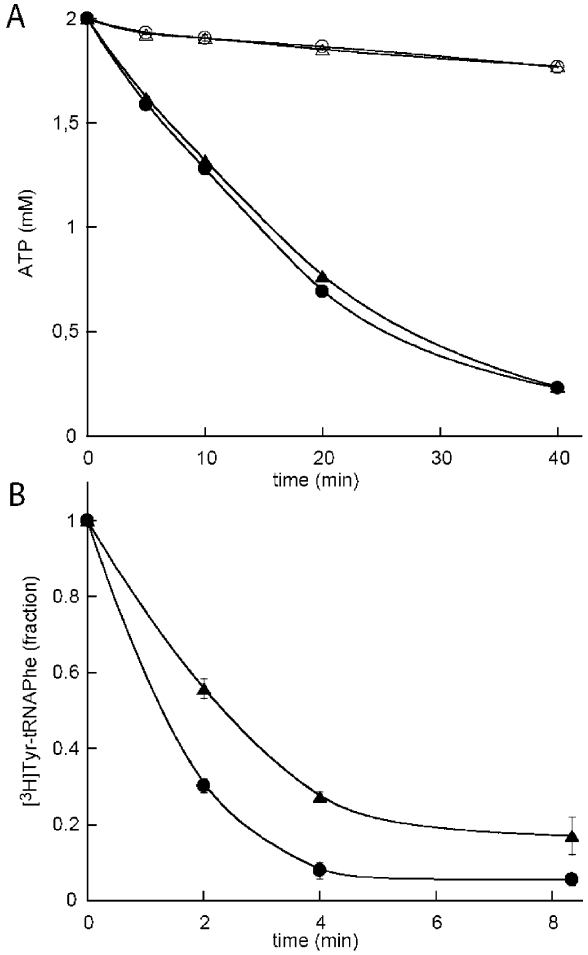

FIGURE 4: Effect of the B2 domain of *E. coli* PheRS upon the editing activity toward Tyr. (A) Cis-editing activity. (B) Trans-editing activity. (● and ○) PheRSαA294G, (▲ and △) PheRSαA294GβΔB2, and (○ and △) no tRNA.

DISCUSSION

The B2 Domain Is a Potential Secondary tRNA-Binding Site. Deletion analysis of PheRS indicated that the B2 structural module is not a primary binding site for cognate tRNA and, consequently, is not essential for phenylalanylation in vitro or in vivo. These findings echo previous studies of the role of homologous domains in other aminoacylation reactions, where the protein Arc1p and the Trpb111-like C-terminal domain of MetRS were both found to be dispensable in vivo (*16*, *17*). While deletion of B2 had little effect on the use of homologous tRNA by PheRS, heterologous yeast tRNA^{Phe} was a significantly poorer substrate for both binding and aminoacylation by the truncated enzyme. This provides functional support for recent modeling studies that suggested that PheRS contains a second tRNA-binding site in addition to the primary site identified from structural studies (*10*, *22*). Previous fast kinetic studies also proposed the existence of a second tRNA-binding site per active site,

Table 2: Dissociation Constants for Full-Length and Truncated *E. coli* PheRS

PheRS	PheRS αA294G		PheRS αA294G$\beta\Delta$B2		
	K_D[a] (nM)	tRNA-binding sites occupancy[b] (%)	K_D[a] (nM)	tRNA-binding sites occupancy[b] (%)	K_D PheRS αA294G/ K_D PheRS αA294G$\beta\Delta$B2
$tRNA^{Phe}$ *E. coli*	160 ± 10	36 ± 5	120 ± 10	44 ± 3	1.3
$tRNA^{Phe}$ *S. cerevisiae*	250 ± 50	47 ± 9	370 ± 100	29 ± 3	0.7

[a] K_D values were calculated by fitting the data shown in Figure 5 to the quadratic equation for a single binding site. [b] Ratio of total binding sites measured by tRNA titration over the total binding site as determined by active-site titration.

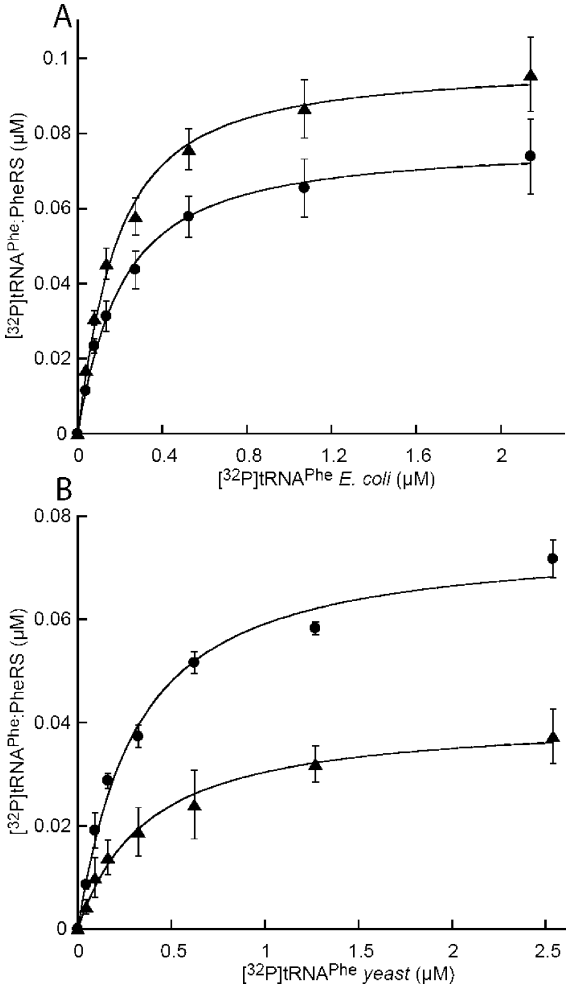

FIGURE 5: Saturation curves for tRNA binding by PheRSαA294G and PheRSαA294G$\beta\Delta$B2. PheRSαA294G (0.3 μM) (●) or PheRSαA294G$\beta\Delta$B2 (0.3 μM) (▲) were mixed with in vitro transcribed $tRNA^{Phe}$ from *E. coli* (A) or yeast (cytosolic) (B).

increasing to four the total number of tRNA-binding sites at the surface of the functional heterotetramer (*25*, *33*, *34*). This second tRNA-binding site was proposed to be involved in promoting the dissociation of the product, Phe-$tRNA^{Phe}$, from the active site. In light of these previous observations and the data obtained here, it is plausible that the B2 domain is a secondary tRNA-binding site. Attempts to provide further support for the existence of a second functional tRNA-binding site using fast kinetic techniques have, to date, proven to be unsuccessful because of the absence of a significant change in fluorescence quenching resulting from the interaction between PheRS and in vitro transcribed $tRNA^{Phe}$ (H. Roy and M. Ibba, unpublished results).

Contribution of the B2 Domain to Post-transfer Editing Activity. An appreciable reduction in the rate of trans editing of Tyr-$tRNA^{Phe}$ was observed for PheRSαA294G$\beta\Delta$B2 compared to full-length PheRS, in contrast to the more modest effects of this deletion on aminoacylation. The decrease in the trans-editing rate of Tyr-$tRNA^{Phe}$ and the slightly improved affinity for homologous tRNA measured with PheRSαA294G$\beta\Delta$B2 support the above proposal that the B2 domain might participate in the dissociation of aminoacyl-tRNA from the active site. We recently found that trans editing of Tyr-$tRNA^{Phe}$ is faster than the aminoacylation reaction, which, given that aminoacyl-tRNA dissociation is not rate-limiting (*35*), suggests that the rate of trans editing approximates that of cis editing (J. Ling, H. Roy, and M. Ibba, unpublished results). Thus, the reduced rate of editing by PheRSαA294G$\beta\Delta$B2 likely results from changes in aminoacyl-tRNA binding and translocation rather than hydrolysis of the mischarged tRNA. When these findings are taken together, they suggest that the role of the B2 domain may be to draw aminoacyl-tRNAs away from the synthetic active site toward the editing site. In the case of cognate Phe-$tRNA^{Phe}$, which is excluded from the editing site, the net result will be to promote product dissociation from PheRS. Because product dissociation from PheRS is not rate-limiting (*35*), deletion of B2 would therefore be expected to have no significant effect on the observed rate of aminoacylation. In contrast, noncognate Tyr-$tRNA^{Phe}$ is the primary substrate of the editing site, and deletion of B2 would be expected to have a deleterious effect on the observed rate of trans editing, as was the case here.

The EMAPII Protein Fold Acts by Modulating tRNA Binding. Our data suggest that the function of the B2 domain of PheRS is to control aminoacyl-tRNA dissociation from the active site by providing a second distal lower affinity tRNA-binding site. This proposed role is consistent with recent in silico studies indicating how the electrostatic potential of the B2 domain could modulate long-range interactions with tRNA (*22*). Such a mechanism, where an EMAPII domain modulates RNA binding rather than acting as the primary determinant of its specificity, has now been described in several proteins including Arc1p, MetRS, and Trbp111 (*16*, *18*, *19*, *36*). In all of these examples and in PheRS, the high-resolution structures of the EMAPII domains are highly conserved. This high degree of evolutionary conservation in the EMAPII domain initially seems at odds with the fact that it is generally dispensable for viability. Early studies of the Arc1p protein showed that, while it is not essential for growth, it does contribute to fitness (*17*), suggesting that the EMAPII domain offers the potential to fine-tune tRNA-binding activity of proteins in response to as yet undefined physiological constraints.

ACKNOWLEDGMENT

We thank D. Tirrell and O. Uhlenbeck for strains and plasmids and V. Godinic, C. Hausmann, J. Levengood, J.

Ling, and S. Wang for critical reading of the manuscript.

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BI060549W