

Anticodon Domain Methylated Nucleosides of Yeast tRNA^{Phe} Are Significant Recognition Determinants in the Binding of a Phage Display Selected Peptide[†]

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ABSTRACT: The contributions of the natural modified nucleosides to RNA identity in protein/RNA interactions are not understood. We had demonstrated that 15 amino acid long peptides could be selected from a random phage display library using the criterion of binding to a modified, rather than unmodified, anticodon domain of yeast tRNA^{Phe} (ASL^{Phe}). Affinity and specificity of the selected peptides for the modified ASL^{Phe} have been characterized by fluorescence spectroscopy of the peptides' tryptophans. One of the peptides selected, peptide t^F2, exhibited the highest specificity and most significant affinity for ASL^{Phe} modified with 2'-O-methylated cytidine-32 and guanosine-34 (Cm₃₂ and Gm₃₄) and 5-methylated cytidine-40 (m⁵C₄₀) ($K_d = 1.3 \pm 0.4 \mu\text{M}$) and a doubly modified ASL^{Phe}-Gm₃₄,m⁵C₄₀ and native yeast tRNA^{Phe} ($K_d \cong 2.3$ and $3.8 \mu\text{M}$, respectively) in comparison to that for the unmodified ASL^{Phe} ($K_d = 70.1 \pm 12.3 \mu\text{M}$). Affinity was reduced when a modification altered the ASL loop structure, and binding was negated by modifications that disfavored hairpin formation. Peptide t^F2's higher affinity for the ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ hairpin and fluorescence resonance energy transfer from its tryptophan to the hypermodified wybutosine-37 in the native tRNA^{Phe} placed the peptide across the anticodon loop and onto the 3'-side of the stem. Inhibition of purified yeast phenylalanyl-tRNA synthetase (FRS) catalyzed aminoacylation of cognate yeast tRNA^{Phe} corroborated the peptide's binding to the anticodon domain. The phage-selected peptide t^F2 has three of the four amino acids crucial to G₃₄ recognition by the β -structure of the anticodon-binding domain of *Thermus thermophilus* FRS and exhibited circular dichroism spectral properties characteristic of β -structure. Thus, modifications as simple as methylations contribute identity elements that a selected peptide specifically recognizes in binding synthetic and native tRNA and in inhibiting tRNA aminoacylation.

RNA interaction with proteins is fundamental to numerous biological processes and results in the controlled expression of genes (1). The importance of the posttranscriptional modifications to RNA function is irrefutable (2–10). In all organisms, a very large investment in genes, energy, and material resources evolved to accomplish the 100 chemically and structurally different, site-specific modifications of the RNA nucleobases and ribose (5–7). Modifications are as simple as methylations or as complex as thiolations, glycosidic bond substitutions, and amino acid additions. Modified nucleosides contribute to nucleic acids the same three broadly based chemical interactions that amino acid side chains contribute to proteins: i.e., hydrophobic, hydrophilic, and electrostatic (7). However, the physicochemical contributions

of the modified nucleosides as identity elements for protein recognition are not understood.

Modified-nucleoside-dependent, protein recognition of RNA has never been studied in detail because of the technical difficulties inherent in the synthesis and study of entire RNA molecules with site-specifically incorporated, single and multiple modifications. However, studies of tRNA with its many modifications and known biochemistry have revealed that tRNA modifications are recognition determinants for aminoacyl-tRNA synthetases (aaRS) and initiation and elongation factors (5, 6). Though anticodon domain modified nucleosides are known to be crucial for the recognition of cognate tRNA by some aaRSs and are accepted as identity elements of tRNAs, the range of physicochemical contributions to this recognition is not understood. For instance, *Escherichia coli* ERS (class I synthetase) requires modification of wobble position U₃₄ for aminoacylation of the tRNA (11, 12), whereas yeast FRS (class II synthetase) activity is only moderately enhanced by the presence of the five anticodon domain modifications (13, 14). A single methyl group of 1-methylguanosine-37, m¹G₃₇,¹ prevents misarginylation of tRNA^{Asp} by yeast RRS (15), and the presence of the modified nucleoside lysidine within the anticodon of *E. coli* tRNA^{Ile} eliminates mismethionylation by MRS (16).

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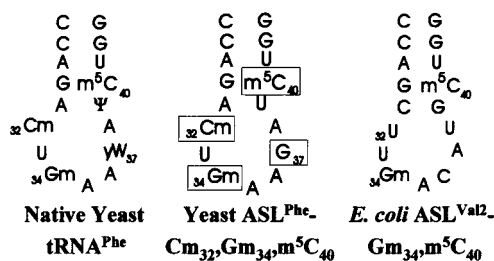


FIGURE 1: Sequences and secondary structures of the native yeast tRNA^{Phe} anticodon domain and synthetic ASLs. The nucleoside sequence shown (left) is that of the tRNA^{Phe} anticodon stem and loop domain with its five naturally occurring modifications and that of the chemically synthesized, triply modified ASL^{Phe} used to select phage from the phage display library. Unmodified and variously modified ASL^{Phe} molecules were synthesized with nucleoside substitutions individually and in combinations according to the natural modifications of yeast tRNA^{Phe}: Cm at position 32, Gm at position 34, m¹G at position 37 (in lieu of yW), and m⁵C at position 40. The combined incorporation of Cm₃₂ with Gm₃₄ produced an RNA with preference to form a duplex rather than a hairpin (29). Also shown is the nucleoside sequence of the designed and synthesized ASL^{Val2} (right). The anticodon domain of native *E. coli* tRNA^{Val2} is devoid of modified nucleosides and has the unmodified and reverse orientation of the G₃₀•C₄₀ base pair designed for ASL^{Val2}.

These observations of modification-influenced, protein recognition have not been equated to the known impact of modifications on the local and global conformations of the anticodon domain (17–19) and the TΨC domain (20, 21). AARS recognition and discrimination of anticodon domains appear to be the function of relatively small regions of these proteins; a peptide as small as 10 amino acids in length is sufficient to confer aaRS specificity (22, 23).

Therefore, we postulated that a well-defined, small model system could allow for a manageable approach to understanding the chemical and physical contributions of individual and multiple RNA modifications to protein recognition and abrogate the complexities of studying modified nucleoside contributions to a macromolecular interaction of RNA with protein (24). The first experimental model would be composed of a well-characterized, chemically synthesized, biologically active (25, 26), modified domain of a thoroughly investigated RNA, yeast tRNA^{Phe} (17, 25, 27, 28), and a peptide selected for its specificity and affinity for the modified RNA. The anticodon stem and loop domain, ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀, with three of the five native modified nucleosides (Figure 1), was used for the selection of peptides (24). Here, we report that at least one of the selected peptides recognizes specific modified nucleosides and its binding of RNA is conformation dependent. We describe the binding affinities and specificities of the peptides selected from the RPL.

MATERIALS AND METHODS

Chemical Synthesis, Purification, and Quality Analyses of Unmodified and Modified RNAs. The native, fully modified yeast tRNA^{Phe} anticodon stem and loop contain 2'-O-methyl

C₃₂ and G₃₄ (Cm₃₂ and Gm₃₄), a tricyclic derivative of G, wybutosine (yW₃₇), pseudouridine (Ψ₃₉), and 5-methyl C₄₀ (m⁵C₄₀) (Figure 1). For this study, heptadecamers representing the anticodon stem and loop of the yeast tRNA^{Phe}, ASL^{Phe}, nucleoside sequence were synthesized on a Perkin-Elmer Applied Biosystems Model 394 automated synthesizer using standard RNA phosphoramidite method (29). The four standard nucleoside phosphoramidites and two additional modified phosphoramidites, Cm and Gm, were purchased from Glen Research (Sterling, VA). The phosphoramidite of m⁵C was obtained from ChemGene (Waltham, MA). Triply modified ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ and quadruply modified ASL^{Phe}-Cm₃₂,Gm₃₄,m¹G₃₇,m⁵C₄₀ were synthesized with TOM chemistries (Xeragon AG, Zürich) for A, C, G, and U and standard phosphoramidite chemistries for the modified nucleosides. The protected m¹G nucleoside phosphoramidite was a gift from Drs. J. Jankowska and A. Kraszewski (Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland). RNAs were synthesized with standard automated ribonucleoside phosphoramidite chemistries (30) altered for modified nucleosides (29). All oligonucleotides were purified by anion-exchange HPLC using a Machery-Nagel (Duren, Germany) 250/10 Nucleogen DEAE 60-7 column. The purity of each oligonucleotide was confirmed by polyacrylamide gel electrophoresis in 7 M urea with 20% acrylamide and nucleoside composition (29). As determined from polyacrylamide gel electrophoresis and UV-monitored thermal denaturations of all the ASLs and NMR spectroscopy of ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ and ASL^{Phe}-Cm₃₂,Gm₃₄,m¹G₃₇,m⁵C₄₀, all of the ASLs adopted unimolecular, hairpin structures under native conditions except ASL^{Phe}-Cm₃₂,Gm₃₄. ASL^{Phe}-Cm₃₂,Gm₃₄ and a 33 nucleoside RNA migrated similarly in polyacrylamide gel electrophoresis under native conditions (supplementary figure; see Supporting Information). Also, the melting temperature of ASL^{Phe}-Cm₃₂,Gm₃₄, derived from thermal denaturations, was concentration dependent. Thus, ASL^{Phe}-Cm₃₂,Gm₃₄ exhibited properties attributable to RNA duplexes. Yeast tRNA^{Phe} was purchased (Sigma, St. Louis, MO).

Peptide Synthesis, Purification, and Quality Analysis. Three 15 amino acid peptide sequences were identified from the library screened with triply modified ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ (24). Because of the potential of selecting peptides against other tRNAs, and other RNAs in general, the peptides were designated as t^F2, t^F3, and t^F33 (Table 1) to denote their binding of tRNA^{Phe}. Peptide t^F0 (Table 1) was a negative control for tRNA–peptide binding. All four peptides were synthesized on a Labortec AG model SP peptide synthesizer using the 9-fluorenylmethoxycarbonyl (Fmoc) procedure on a Tenta Gel S AC resin (capacity 0.28 mmol/g). C-Terminal residues were attached to the resin using the symmetrical anhydride of the protected amino acid in the presence of a catalytic amount of 4-(dimethylamino)pyridine (DMAP). Couplings of 1.5 h were achieved with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetraborate (TBTU)/N-hydroxybenzotriazole (HOBt)/N,N-diisopropylethylamine (DIEA) (1:1:2) in N,N-dimethylformamide (DMF):N-methylpyrrolidine (NMP) (1:1 v/v). Deprotection was accomplished with 20% piperidine in DMF at 25 °C. Peptides were cleaved from resin with trifluoroacetic acid (TFA)/phenol/triisopropylsilane (TIS)/water (88:5:2:5 v/v/v/v) for 2 h at 25 °C. The peptides were purified by column

¹ Abbreviations: ASL, anticodon stem and loop; Cm, 2'-O-methylcytosine; Gm, 2'-O-methylguanosine; m⁵C, 5-methylcytidine; yW, wybutosine; m¹G, 1-methylguanosine; Ψ, pseudouridine; RP-HPLC, reverse-phase high-performance liquid chromatography; CD, circular dichroism; FRET, fluorescence resonance energy transfer; RPL, random peptide library.

Table 1: Binding of RPL-Selected Peptides to ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ and Unmodified ASL^{Phe}

peptide	sequence	frequency in clones (%)	fluorescence quench (max %) ^a	<i>K_d</i> (μM)	
				ASL ^{Phe} -Cm ₃₂ ,Gm ₃₄ ,m ⁵ C ₄₀	unmodified ASL ^{Phe}
t ^F 2	S ¹ ISPW ⁵ GFSGL ¹⁰ LRWSY ¹⁵	40 ^b	80 ± 2	1.3 ± 0.4	70.1 ± 12.3
t ^F 3	A ¹ AGGF ⁵ FRWIF ¹⁰ SPVYV ¹⁵	5 ^b	80 ± 3	10.3 ± 2.2	59.8 ± 6.1
t ^F 33	L ¹ RYTL ⁵ ETRWA ¹⁰ VLSVY ¹⁵	1	75 ± 4	33.4 ± 9.1	47.9 ± 4.2
t ^F 0	S ¹ WHQW ⁵ IWPSG ¹⁰ QPLSE ¹⁵		74 ± 3	45.4 ± 6.4	45.1 ± 4.4

^a Fluorescence quenching by addition of saturating amounts of ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀. ^b Percent of selected phage clones having the sequence out of a total of 200 clones.

chromatography with a preparative RP-HPLC Vydac C18 column (32 × 240 mm, 15–20 μm particle size). Peptides t^F2 and t^F3 were eluted with a linear gradient (25–40%) of acetonitrile (ACN) in 0.1% TFA, peptide t^F0 was eluted with isocratic ACN (22%) in 0.1% TFA, and peptide t^F33 was eluted with a linear gradient of ACN (10–40%) in 0.1% TFA. Elution was conducted at a flow rate of 18 mL/min. The chromatography was monitored at an absorbance of 226 nm. Peptide purity was characterized by analytical HPLC with a System Gold Beckman chromatograph using a Vydac C18 column [4.6 × (45 + 250) mm, 5 μm particle size] and a linear gradient elution of 20–80% ACN in 0.08% TFA (1 mL/min) monitored at 226 nm. The recorded retention times for the four peptides were *R_t*(t^F2) = 19.70 min, *R_t*(t^F3) = 21.67 min, *R_t*(t^F33) = 16.50 min, and *R_t*(t^F0) = 15.67 min. Peptide molecular ions were determined using fast atom bombardment mass spectrometry (FAB MS) with an AMD-604 mass spectrometer. The calculated peptide molecular weights in comparison to their molecular ions (M + H)⁺ were as follows: t^F2, calcd 1756.0, found 1756.4; t^F3, calcd 1717.0, found 1717.6; t^F33, calcd 1871.2, found 1871.5; t^F0, calcd 1838.0, found 1838.7.

Tryptophan Fluorescence Quenching and Fluorescence Resonance Energy Transfer (FRET). The ability of the peptides to bind different RNA sequences was characterized by monitoring the changes in the intrinsic fluorescence properties of tryptophan residues upon the addition of RNA. Tryptophan fluorescence was monitored with a QuantaMaster Model C 61 spectrofluorometer (Photon Technology International Inc.). The titrations were performed at 25 °C with varying amounts of RNA added to a fixed peptide concentration of 1.3 μM in 400 μL of 10 mM sodium cacodylate buffer of pH 6.4, unless specified otherwise. Peptide fluorescence was measured at 340 nm emission (290 nm excitation) using a 1 cm cuvette path length. Experiments were repeated three times with fluorescence detection collected over 10 s after an initial 0.5 min period of equilibration. To assess and correct for inner-filter effects and background, fluorescence intensities were adjusted for dilution, buffer fluorescence, and screening effects due to the presence of RNA. The RNAs inner-filter effect was minimal, and the buffer chosen, cacodylate, had insignificant effects on peptide tryptophan fluorescence. Photobleaching was determined to be only 1–2% of total fluorescence over a period of 10 min. Changes in peptide fluorescence at 340 nm during titration with an ASL or native tRNA^{Phe} generated binding curves from which dissociation constants (*K_d*) were determined using a single binding site curve-fitting procedure implemented in Prism software (GraphPad Software Inc., San Diego, CA). FRET experiments were conducted between the tryptophans of peptide t^F2 and wybutosine (yW) at position 37 in native

yeast tRNA^{Phe}. The absorption and emission spectra of peptide t^F2 (λ_{abs} = 290 nm; λ_{ems} = 340 nm) and yeast tRNA^{Phe} (λ_{abs} = 320 nm; λ_{ems} = 420 nm) were collected under the conditions in which peptide binding was observed. The yW emission spectra were collected for yeast tRNA^{Phe} in the presence and absence of RNA-binding peptides and control peptides at 25 °C. Peptide t^F2 and tRNA concentrations were equimolar (1.3 μM in 10 mM sodium cacodylate, pH 6.4).

Aminoacylation Inhibition Assays. Purified yeast phenylalanyl-tRNA synthetase (FRS) was a gift of Drs. Alexey D. Wolfson and Olke Uhlenbeck (31). L-[4-³H]Phenylalanine (15–30 Ci/mmol) was purchased (Amersham Pharmacia Biotech). Aminoacylation reactions (50 μL) were performed in reaction buffer (50 mM HEPES, pH 7.5, 25 mM KCl, 15 mM MgCl₂, 0.1 mM DTT, 2 mM ATP, 5 μM L-[4-³H]-phenylalanine). The concentration of yeast tRNA^{Phe} was 0.5 μM. In reactions containing peptides, the indicated peptides (10 μM) were incubated with tRNA^{Phe} in the reaction buffer on ice for 30 min. FRS was added to start the reaction at a concentration of 1 nM and incubated at 25 °C. Aliquots (5 μL) were removed at time points (0.5, 1.0, 2.0, 3.0, and 5.0 min) and spotted onto Whatman 3MM filter paper. The filters were placed into a 10% trichloroacetic acid solution, washed in 5% trichloroacetic acid solution, and air-dried, and the radioactivity on the filters was assessed by scintillation counting. Parallel experiments were conducted in the absence of enzyme. Background (in the absence of FRS) at each time point was subtracted. Picomoles of incorporated phenylalanine was plotted against time, and data were fitted to a single binding site curve-fitting procedure implemented in Prism software. Picomoles of phenylalanine incorporated was determined from the counting efficiency of a [³H]phenylalanine standard under the same conditions as those of the experimental samples.

Circular Dichroism Spectropolarimetry (CD). CD spectra of the four peptides were recorded on a Jasco 600 spectropolarimeter. CD spectra were collected for samples of peptide concentration 22.4 μM (10 mM sodium cacodylate, pH 6.4) in 0.1 cm path length cuvettes and measured from 196 to 250 nm at 25 °C. Spectra were averaged during five scans. Observed ellipticity (mdeg) was converted to mean molar residue ellipticity (deg cm² dmol⁻¹) per amino acid residue.

RESULTS

Affinity of RPL-Selected Peptides for the Target-Modified ASL^{Phe}. Previously, we demonstrated that peptides selected from a random 15 amino acid phage display library (RPL) bound the modified RNA used for their selection, ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ (24, 32; Figure 1). Qualitative results,

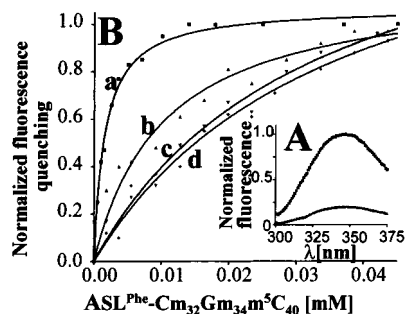


FIGURE 2: Interactions of ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ with peptides t^F2, t^F3, t^F33, and t^F0 monitored with fluorescence spectroscopy. (A, inset) Fluorescence emission spectra of peptide t^F2. Emission spectra of peptide t^F2 (1.3 μM) were collected in the absence (■) and presence (▲) of a saturating amount of ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ (11 μM). Excitation was conducted at 290 nm, and spectra were corrected by subtracting a buffer blank. (B) Fluorescence titration of the RPL-selected peptides with ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀. Peptides t^F2 (a, ■), t^F3 (b, ▲), t^F33 (c, ▼), and t^F0 (d, ◆) were titrated with the ASL^{Phe}. The normalized fluorescence quenching (as a percent of a total fluorescence quench) was plotted against the RNA concentration. Peptide fluorescence was measured at 340 nm emission.

obtained from studies using circular dichroism (CD) (24) and capillary electrophoresis (32), suggested that the peptides bound the ASL^{Phe} with different affinities. The three peptides with highest affinities for ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ had tryptophans in their sequences (Table 1). Therefore, the ASL^{Phe} binding of the three selected peptides, and that of a negative control peptide (t^F0, Table 1) with a tryptophan, was characterized by monitoring changes in tryptophan fluorescence at 340 nm. In the presence of ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀, the fluorescence emission of all four peptides was reduced (Table 1). Fluorescence of the peptide with highest affinity, t^F2, was quenched 80% at a ratio of RNA to peptide of 8/1 (Figure 2A; Table 1). The fluorescence quenching of the peptides with saturating amounts of ASL^{Phe} varied between 70% and 80%. Affinities of each peptide for ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ were determined from binding curves generated by titrating each peptide with increasing amounts of RNA and monitoring the change in fluorescence (Figure 2B). Peptide t^F2 bound ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ with the highest affinity ($K_d = 1.3 \pm 0.4 \mu\text{M}$) (Table 1). Peptide t^F2 bound ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ with a stoichiometry of 1:1, determined from binding isotherms with a concentration of peptide as high as 10-fold the K_d (supplementary figure; see Supporting Information). In addition, the RNA was observed to form only a single, 1:1 complex with peptide t^F2 when monitored by capillary electrophoresis (supplementary figure; see Supporting Information). A lower affinity was recorded for peptide t^F3 ($K_d = 10.3 \pm 2.2 \mu\text{M}$). Peptides t^F33 and t^F0 bound ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ with considerably reduced affinities compared to that of t^F2 ($K_d = 33.4 \pm 9.1$ and $45.4 \pm 6.4 \mu\text{M}$, respectively) (Figure 2B and Table 1).

Modified Nucleoside Recognition Determinants. The importance of modified nucleosides to peptide recognition of the ASL and to the specificity of that recognition was assessed. Peptides t^F2, t^F3, t^F33, and t^F0 exhibited differentially reduced affinities for the unmodified ASL^{Phe} in comparison to that for ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ (Table 1). The peptide that bound ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ most

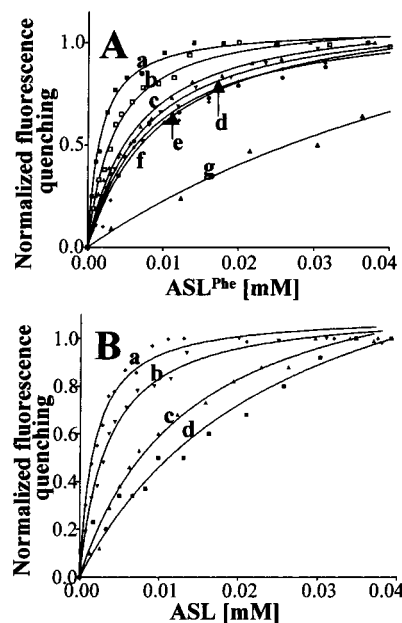


FIGURE 3: Titrations of peptide t^F2 with variously modified ASL^{Phe}, tRNA^{Phe}, and *E. coli* ASL^{Val2}. (A) Peptide t^F2 titrated with variously modified ASL^{Phe}. To determine which of the modified nucleosides were important recognition determinants for peptide t^F2, the peptide was titrated with variously modified ASL^{Phe}, and the changes in fluorescence were monitored: (a) ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ (a, ■); (b) ASL^{Phe}-Gm₃₄,m⁵C₄₀ (b, □); (c) ASL^{Phe}-m⁵C₄₀ (c, ▲); (d) ASL^{Phe}-Gm₃₄ (d, ▼); (e) ASL^{Phe}-Cm₃₂ (e, ◆); (f) ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ (f, ●); (g) unmodified ASL^{Phe} (g, △). (B) Peptide t^F2 titrated with native tRNA^{Phe} and ASL^{Val2}. Changes in tryptophan fluorescence were monitored under the experimental conditions described in Materials and Methods and Figure 2: (a) tRNA^{Phe} (a, ◆); (b) ASL^{Phe}-Gm₃₄,m⁵C₄₀ (b, ▼); (c) ASL^{Val2}-Gm₃₄,m⁵C₄₀ (c, ▲); (d) unmodified ASL^{Val2} (d, ■).

tightly, t^F2 ($K_d = 1.3 \pm 0.4 \mu\text{M}$), bound the unmodified ASL^{Phe} with the least affinity ($K_d = 70.1 \pm 12.3 \mu\text{M}$; Table 1). Peptide t^F3's affinity for the triply modified ASL^{Phe} was 6-fold its affinity for the unmodified ASL^{Phe} (Figure 3). In contrast, there were only small differences between the affinities of peptides t^F33 and t^F0 for ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ and their recognition of the unmodified ASL^{Phe} (Table 1). Interestingly, the affinities of the peptides for the ASL^{Phe} correlated with their presence in the 200 phage that were selected and sequenced (Table 1). In comparing the dissociation constants for the four peptides for the triply modified ASL^{Phe} and the unmodified ASL^{Phe} (Table 1), we concluded that the modified ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ was recognized specifically by t^F2 and t^F3, but that t^F33 and the negative control peptide t^F0 had exhibited nonspecific associations with the two ASLs.

Of the two peptides that demonstrated specificity for the modified ASL^{Phe}, peptide t^F2 had the highest affinity. Therefore, we investigated the importance of individual nucleoside modifications and combinations of those modified nucleosides for peptide t^F2 recognition of the ASL^{Phe} (Figure 3). All singly modified ASL^{Phe} constructs were recognized by peptide t^F2 with affinities 5–10-fold stronger than the peptide's affinity for the unmodified ASL^{Phe}. However, t^F2 interaction with each of the singly modified ASL^{Phe} was considerably weaker than that for ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ (Table 2). The associations of singly modified ASL^{Phe}-Gm₃₂ and ASL^{Phe}-m⁵C₄₀ with peptide t^F2 were similar, $K_d = 7.6$

Table 2: Interaction of Various Modified ASL^{Phe} and Native Yeast tRNA^{Phe} with Peptide t^{F2}

ASL or tRNA	buffer ^a conditions	K _d (μM)
ASL ^{Phe} -Cm ₃₂ ,Gm ₃₄ ,m ⁵ C ₄₀	10 mM, pH 6.4	1.3 ± 0.4
	100 mM, pH 6.4	2.9 ± 0.5
	1 mM MgCl ₂	5.6 ± 0.7
	10 mM MgCl ₂	5.1 ± 1.1
	25 mM KCl	1.0 ± 0.2
	50 mM, pH 7.4, 25 mM KCl, 15 mM MgCl ₂	0.2 ± 0.1
ASL ^{Phe} -Cm ₃₂	50 mM, pH 7.4	0.2 ± 0.1
	10 mM, pH 5.5	3.2 ± 0.5
ASL ^{Phe} -Gm ₃₄	10 mM, pH 6.4	11.0 ± 1.9
ASL ^{Phe} -m ⁵ C ₄₀	10 mM, pH 6.4	7.6 ± 0.6
ASL ^{Phe} -Cm ₃₂ ,Gm ₃₄	10 mM, pH 6.4	6.4 ± 0.5
ASL ^{Phe} -Gm ₃₄ ,m ⁵ C ₄₀	10 mM, pH 6.4	>500
ASL ^{Phe} -Cm ₃₂ ,Gm ₃₄ ,m ⁵ C ₄₀	10 mM, pH 6.4	3.8 ± 0.7
ASL ^{Phe} -Cm ₃₂ ,Gm ₃₄ ,m ¹ G ₃₇ ,m ⁵ C ₄₀	10 mM, pH 6.4	13.3 ± 2.4
yeast tRNA ^{Phe}	10 mM, pH 6.4	2.3 ± 0.4
	10 mM, pH 5.5	1.8 ± 0.2
<i>E. coli</i> ASL ^{Val2}	10 mM, pH 6.4	25.4 ± 6.3
<i>E. coli</i> ASL ^{Val2} -Gm ₃₄ ,m ⁵ C ₄₀	10 mM, pH 6.4	14.1 ± 2.2

^a Sodium cacodylate buffer.

± 0.6 μM and 6.4 ± 0.5 μM, respectively. However, the singly modified ASL^{Phe}-Cm₃₂ was bound with a slightly lower affinity ($K_d = 11.0 \pm 1.9 \mu\text{M}$). These results indicated a fundamental involvement of modified nucleosides in ASL-peptide recognition and showed that two of the modifications, Gm₃₄ and m⁵C₄₀, may be more important than the third, Cm₃₂. To confirm the importance of Gm₃₄ and m⁵C₄₀ for ASL recognition and binding by peptide t^{F2}, we investigated the binding of the doubly modified ASL^{Phe}-Gm₃₄,m⁵C₄₀. The association of doubly modified ASL^{Phe}-Gm₃₄,m⁵C₄₀ for peptide t^{F2} was about 2-fold higher than that of the singly modified ASL^{Phe}-m⁵C₄₀, but still about one-fourth that of the triply modified ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ (Table 2). In contrast to the doubly modified ASL^{Phe}-Gm₃₄,m⁵C₄₀, peptide t^{F2} had little to no affinity for the doubly modified ASL^{Phe}-Cm₃₂,Gm₃₄ ($K_d > 500 \mu\text{M}$). This observation is explained by the fact that ASL^{Phe}-Cm₃₂,Gm₃₄ does not adopt a stable stem-loop structure but instead adopts a structure with characteristics attributable to a duplex under the conditions of the assay (25).

To determine if the properties of Gm₃₄ and m⁵C₄₀ that are important for peptide t^{F2} recognition of ASL^{Phe} were transferable to another tRNA sequence, we synthesized modified and unmodified analogues of the anticodon domain of *E. coli* tRNA^{Val2} (ASL^{Val2}). The anticodon loop of ASL^{Val2} has only two nucleosides, G₃₄ and A₃₅, as well as the invariant U₃₃ in common with yeast and other tRNA^{Phe} and is naturally devoid of modifications (Figure 1) (33). Though the base pairs in the upper part of the ASL stem are similar to that in yeast tRNA^{Phe}, the sequences in the lower stem and loop are quite different. Wobble position 34 is a guanosine residue that was modified in our altered version of this ASL^{Val2} to Gm₃₄. For introduction of m⁵C₄₀, we inverted the C₃₀•G₄₀ base pair to a G₃₀•m⁵C₄₀ pair (Figure 1). The unmodified native ASL^{Val2} was bound by peptide t^{F2} with a K_d of $25.4 \pm 6.3 \mu\text{M}$ (Table 2). However, the altered ASL^{Val2}-Gm₃₄,m⁵C₄₀ was bound by peptide t^{F2} with a 2-fold higher affinity ($K_d = 14.1 \pm 2.2 \mu\text{M}$) (Table 2). Thus, the two modifications affected the affinity of peptide t^{F2} for RNA.

The anticodon stem and loop of native yeast tRNA^{Phe} are modified with wybutosine-37 (yW₃₇), a ring and side chain addition to G₃₇, and Ψ₃₉, in addition to Cm₃₂, Gm₃₄, and m⁵C₄₀. Native tRNA^{Phe} was bound by peptide t^{F2} with a K_d of $2.3 \pm 0.4 \mu\text{M}$ (Table 2) which compared favorably to that of the triply modified ASL^{Phe} (Table 2). Because the naturally occurring yW₃₇ is unstable to the conditions of automated RNA synthesis, m¹G, the natural precursor of yW (34), was introduced at position 37 of ASL^{Phe}. However, the affinity of peptide t^{F2} for the quadruply modified ASL^{Phe}-Cm₃₂,Gm₃₄,m¹G₃₇,m⁵C₄₀ was considerably reduced ($K_d = 13.3 \pm 2.4 \mu\text{M}$) compared to that for the triply modified ASL (Figure 3; Table 2). The reduced affinity of peptide t^{F2} for ASL^{Phe}-Cm₃₂,Gm₃₄,m¹G₃₇,m⁵C₄₀ as compared to that of the triply modified ASL could be the result of m¹G₃₇ altering the anticodon loop conformation. A comparison of NMR-derived solution structures of ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ and ASL^{Phe}-Cm₃₂,Gm₃₄,m¹G₃₇,m⁵C₄₀ indicates that the only conformational differences occur in and around the anticodon triplet (J. W. Stuart, K. Koshlap, R. Guenther, and P. F. Agris, unpublished). The contrast in peptide t^{F2}'s affinities for native tRNA^{Phe} and that of the quadruply modified ASL^{Phe} also could be explained by differences in anticodon loop structure.

Effects of Solution Conditions on Modified ASL^{Phe}/Peptide Affinity. The influence of ionic strength and pH on the association of peptide t^{F2} for the triply modified ASL^{Phe} and native tRNA^{Phe} was investigated in order to obtain an indication of the forces that may be involved in binding. The ionic strength dependence of peptide t^{F2}'s interaction with ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ was evaluated by varying the sodium cacodylate buffer concentration from 10 to 100 mM and by adding either MgCl₂ or KCl (Table 2). A 10-fold increase in the buffer's ionic strength was required to decrease the peptide's affinity by more than 50%. However, the observed change in affinity was modest in comparison to the peptide's reduced affinity for the unmodified ASL (see below). The introduction of 1 and then 10 mM Mg²⁺ to the binding experiment reduced t^{F2}'s affinity for the ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ by 50%, similar to that of increasing the monovalent ion concentration 10-fold (Table 2). Under the ionic and pH conditions for aminoacylation of yeast tRNA^{Phe} by purified yeast phenylalanyl-tRNA synthetase (FRS) (50 mM buffer, pH 7.4, 25 mM KCl, 15 mM MgCl₂), peptide t^{F2}'s affinity for ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ was significantly increased. However, the peptide's affinity for the ASL under monovalent ion conditions of 25 mM KCl was similar to that in buffer alone (Table 2), whereas at the pH of the aminoacylation reaction (pH 7.4), the peptide's affinity for the ASL was significantly greater than that at pH 6.4 (Table 2). Lowering the pH to 5.5, helpful in observing and identifying imino and amino protons in structure determination of the complex by NMR spectroscopy, did not affect the binding of peptide to the modified ASL or native tRNA^{Phe} (Table 2). These results suggest that hydrophobic, not electrostatic, interactions may play a dominant role in peptide t^{F2} recognition of the triply modified ASL^{Phe}.

Localization of Peptide Binding to Native tRNA^{Phe}. Wybutosine at position 37 in native yeast tRNA^{Phe} is naturally fluorescent. This natural fluorescent marker has been used to characterize the local anticodon loop environment and binding properties of the anticodon loop (35–37). The

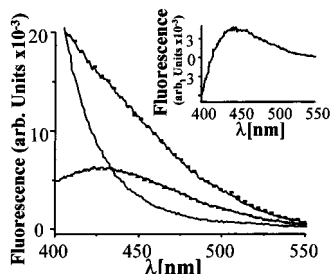


FIGURE 4: Fluorescence resonance energy transfer (FRET) from the tryptophan residues of peptide t^F2 to wybutosine of yeast $tRNA^{Phe}$. The FRET was conducted by excitation of t^F2 at 290 nm. The yeast $tRNA^{Phe}$ yW_{37} emission spectrum was collected in the absence of peptide (\blacktriangle) and in the presence of peptide (\blacksquare). The inset shows a fluorescence difference spectrum that resulted from subtracting the spectra in the absence of peptide from that in the presence of peptide. All spectra were corrected for the fluorescence of the peptide's tryptophan (—).

tryptophan emission spectrum (340 nm maximum) overlaps the absorption spectrum of yW_{37} (320 nm maximum), and the emission of the latter occurs at a higher wavelength (420 nm maximum) than that of tryptophan. Therefore, the binding of peptide t^F2 to the anticodon stem and loop domain of native $tRNA^{Phe}$ should be detectable with a fluorescence resonance energy transfer (FRET). To localize the interaction of peptide t^F2 to the tRNAs anticodon domain, tryptophan and yW_{37} emission spectra were monitored before and after complex formation (Figure 4). The binding of peptide t^F2 to $tRNA^{Phe}$ (at equimolar concentrations) resulted in an increase in yW_{37} fluorescence and a strong quenching ($\sim 40\%$) of the peptide's tryptophan fluorescence. The fluorescence intensity characteristic for yW_{37} after excitation of the $tRNA^{Phe}$ —peptide t^F2 complex at the tryptophan absorbance of 290 nm was about 2-fold higher than that observed for the free $tRNA^{Phe}$ after excitation at 320 nm, the optimum excitation wavelength of yW_{37} . This fluorescence intensity increase occurred only when the excitation of complex was performed at the tryptophan absorption band wavelength and confirmed that the change of fluorescence of yW_{37} in the complex was an effect of the FRET. Native yeast $tRNA^{Phe}$ induced only a small decrease in the tryptophan fluorescence of the control peptide t^F0 , and no $Trp \rightarrow yW_{37}$ FRET was observed (data not shown).

Peptide t^F2 as Inhibitor of $tRNA^{Phe}$ Function. Peptide t^F2 's binding of native $tRNA^{Phe}$ with a $K_d \sim 2 \mu M$ suggested that the peptide could inhibit tRNA function. Aminoacylation and mRNA binding of tRNA at the ribosomal P-site are two functions in which the anticodon participates and are readily assayed in vitro. Attempts to inhibit the binding of $tRNA^{Phe}$ or ASL^{Phe} - $Cm_{32}, Gm_{34}, m^5C_{40}$ to the codon at the ribosomal P-site were unsuccessful perhaps because tRNAs affinity for the P-site is 10-fold higher ($K_d \sim 190$ nM; 25) than the peptide's affinity for the anticodon (supplementary figure; see Supporting Information). The filter-binding assay in which ^{32}P -labeled tRNA or ASL was bound to the *E. coli* 30S ribosomal subunit did not preclude the possibility that a complex of t^F2 and tRNA or ASL was bound to the codon on the ribosome. However, this is an unlikely event because a t^F2 -bound tRNA or ASL would significantly contrast to the known elongation factor presentation of tRNA to the ribosome in which the ASL is completely exposed. The association of yeast phenylalanyl-tRNA synthetase (FRS)

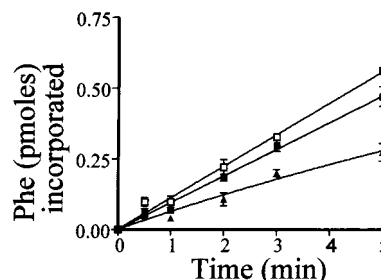


FIGURE 5: FRS-catalyzed aminoacylation of yeast $tRNA^{Phe}$ inhibited in the presence of peptide t^F2 . Purified FRS was used to aminoacylate native yeast $tRNA^{Phe}$ over the course of 5 min. The extent of aminoacylation was monitored in the absence (\blacksquare) and in the presence of peptide t^F2 (\blacktriangle) and negative control peptide t^F0 (\square). The peptide to tRNA ratio was 20/1.

with its cognate tRNA is of the same order ($K_d \sim 1 \mu M$; 13, 38) as that of peptide t^F2 . In addition, the yeast $tRNA^{Phe}$ anticodon nucleosides (Gm_{34} , A_{35} , A_{36}) are recognition determinants for FRS (39). Results from experiments with variously modified ASL^{Phe} and the FRET with native $tRNA^{Phe}$ were strong indicators that peptide t^F2 also may be recognizing the anticodon. Thus, we hypothesized that peptide t^F2 may be able to inhibit the FRS-catalyzed aminoacylation of native $tRNA^{Phe}$, as well as the ASLs binding to the ribosome. Aminoacylation of native yeast $tRNA^{Phe}$ by the purified yeast FRS was inhibited in the presence of peptide t^F2 . At a peptide:tRNA molar ratio of 20:1, the extent of aminoacylation was reduced approximately 45% relative to that in its absence or in the presence of the negative control peptide t^F0 at the same peptide/tRNA ratio (Figure 5).

Peptide t^F2 Secondary Structure. In general, small peptides with sequences of 10–20 amino acids adopt no particular ordered secondary structure in solution. Their CD spectra are usually indicative of random coils. However, some examples of short peptides that do adopt ordered structures, such as β -sheet, have been reported (40–42). To determine if peptides t^F2 , t^F3 , t^F33 , and t^F0 exhibited stable secondary structures in solution in the absence of RNA and whether there was a particular secondary structure correlated with binding affinity for ASL^{Phe} - $Cm_{32}, Gm_{34}, m^5C_{40}$, CD spectra were collected under the conditions used to assess their binding to the ASL^{Phe} (Figure 6). The CD spectra of the peptides were distinguishable, and spectral properties characteristic of secondary structure were evident for peptides t^F2 and t^F33 . Quantification of the peptides' secondary structures was unsuccessful using two different methods, variable selection or VARSELEC (43) and artificial (neural) networks (44). Probably, the CD spectra of these particular peptides do not correspond very well to spectra of the much larger proteins stored in the data banks of the VARSELEC and ANN programs. Though their affinities for the ASL differed by a factor of 30, peptides t^F2 and t^F33 had similar spectral properties characteristic of β -sheet or β -hairpin structures. The CD spectrum of peptide t^F33 was characterized by a distinctive minimum at 216 nm and positive maximum at 199 nm (Figure 6). There was also a positive band of unknown origin centered about 230 nm. The CD spectrum of peptide t^F2 was different than that of peptides t^F3 and t^F33 , particularly with regard to the intensities of the ellipticities. The peptide t^F2 spectrum had a negative

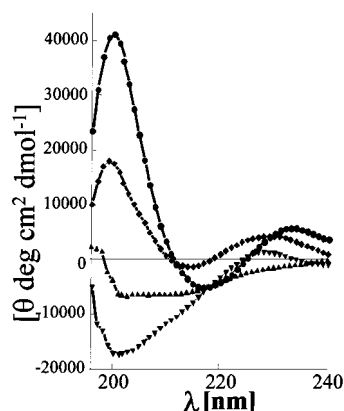


FIGURE 6: CD spectra of peptides t^F2 , t^F3 , t^F33 , and t^F0 . The CD spectra of the four peptides alone were taken under conditions similar to those used in determining affinities for ASL^{Phe} (see Materials and Methods). Peptides: t^F2 (◆); t^F3 (▲); t^F33 (●), and t^F0 (▼). Observed ellipticity (mdeg) was converted to mean molar residue ellipticity $[\Theta]$ (deg cm² dmol⁻¹). All spectra were baseline corrected.

minimum at 213 nm and positive maximum centered at 198 nm, both indicative of β -structure. The reduced intensity and spectral shift of the minimum to a shorter wavelength could be due to the superposition of a distinct negative Cotton effect of a β -sheet structure at 217 nm and moderate Cotton effect of a β -hairpin at 213 nm (45). A broad positive maximum of the CD spectrum of peptide t^F2 centered about 230 nm was probably of structural, not aromatic, amino acid origin because the CD spectrum of a peptide t^F2 analogue, in which glycine-9 was replaced by an alanine ($[A^9]t^F2$), did not display a positive ellipticity above 200 nm (P. Mucha and P. F. Agris, unpublished results). The very broad negative band (198–240 nm) in the CD spectrum of peptide t^F3 may result from a mixture of random coil and β -sheet structures. The spectrum of peptide t^F0 was characteristic of a random coil (Figure 6).

DISCUSSION

Fifteen amino acid long peptides had been selected from an RPL to bind an analogue of the yeast $tRNA^{Phe}$ anticodon stem and loop with three of the five naturally occurring modified nucleosides (24). Using site-specific incorporation of the individual modifications and their combinations, we have characterized the peptide recognition determinants. Modifications, as simple as methylations, contributed identity elements to the synthetic anticodon domain and to the native tRNA. The identity elements contributed by the individual methyl groups or their combinations in the anticodon domain may have produced hydrophobic recognition determinants and/or conformations that were specifically recognized by the RPL selected peptide, t^F2 . Because peptide t^F2 effectively bound $tRNA^{Phe}$ and inhibited its aminoacylation by FRS that recognizes the anticodon, t^F2 must recognize the methyl groups in the $tRNA^{Phe}$ anticodon domain in a context similar to that of ASL^{Phe} -Cm₃₂,Gm₃₄,m⁵C₄₀. Methyl groups incorporated at the same nucleoside residues as ASL^{Phe} , but in the ASL sequence of $tRNA^{Val2}$, were sufficient recognition determinants for peptide t^F2 . We chose *E. coli* $tRNA^{Val2}$ because its anticodon stem and loop sequence was dissimilar to that of yeast $tRNA^{Phe}$ and lacked anticodon domain modifications (33). In addition to these results, a FRET from

t^F2 to the $tRNA^{Phe}$ wybutosine indicated that the peptide binds across the tRNAs anticodon domain. Peptide t^F2 's sequence differs from that of RNA binding motifs but may have anticodon domain binding properties similar to those of FRS.

From the study of individually methylated ASLs, we were able to determine that no single modification was solely responsible for the selectivity of the RNA/peptide binding. Thus, no simple relationship between affinity and modified nucleoside location, loop or stem, in the singly modified ASL^{Phe} was obvious. The individual incorporations of Cm₃₂, Gm₃₄, or m⁵C₄₀ to unmodified ASL^{Phe} increased the binding affinity of peptide t^F2 about 1 order of magnitude. The presence of a single methylation may be a driving force of the recognition process because introduction of both Gm₃₄ and m⁵C₄₀ only slightly improved peptide t^F2 's affinity for the ASL^{Phe} . Even individual methylations produce dramatic effects in tRNA function (m¹G₃₇ in translational frame shifting; 46), metal ion binding (m⁵C₄₀ in Mg²⁺ binding to the ASL^{Phe} ; 7), thermostability (m¹G₃₇ in ASL^{Phe} ; 25) and structure (m⁵U₅₄ in the T Ψ C stem and loop; 21).

In general, the introduction of a methyl group to an RNA enhances a localized hydrophobicity (7). However, depending upon the type of methylation, base carbon or nitrogen or 2'-O-ribose, a variety of RNA physicochemical properties could result in the recognition determinants for peptide t^F2 . The modified nucleosides Cm₃₂ and Gm₃₄ are methylated at the sugar, 2'-oxygen atom and are located in the ASL^{Phe} loop, whereas m¹G₃₇ also located in the loop and m⁵C₄₀ located in the ASL^{Phe} stem are methylated at the nucleobase N1 and C5 atoms, respectively. Substitution of m⁵C₄₀ for C₄₀ increased stacking interactions in the stem of ASL^{Phe} (J. W. Stuart, K. Koshlap, R. Guenther, and P. F. Agris, unpublished) and in a DNA analogue (47), as would be expected for an increased hydrophobicity of the base. In comparison, we found that the G to m¹G₃₇ substitution that reduced peptide t^F2 affinity when introduced into ASL^{Phe} -Cm₃₂,Gm₃₄,m⁵C₄₀ negates an intraloop base pair, increases 3'-side base stacking, and noticeably alters the anticodon loop conformation (J. W. Stuart, K. Koshlap, R. Guenther, and P. F. Agris, unpublished). The ribose 3'-endo sugar pucker of mononucleosides and that of individual residues in oligonucleotides are stabilized by the 2'-O-methyl group (7). However, the 2'-O-methylation also negates noncanonical hydrogen bonding across loops (7). Though the individual 2'-O-methylations resulted in an enhanced affinity of the peptide for the ASL, Cm₃₂ and Gm₃₄ together significantly decreased binding affinity. This doubly modified ASL^{Phe} -Cm₃₂,Gm₃₄ preferentially adopts a structure with properties characteristic of a duplex (25) and may be relevant to the chronology of RNA modifications and processing events in tRNA (5, 6, 48) and rRNA (49). We can conclude that the site-specific methyl groups are recognition determinants for t^F2 binding to the ASL^{Phe} and native $tRNA^{Phe}$ but that the structural context in which they occur is equally important.

Insight into the chemical character of the ASL^{Phe} -peptide interaction resulted from investigating the effects of ionic strength and pH change on peptide t^F2 affinity for the ASL and the native tRNA. The effects of ionic strength (Table 2) suggest that hydrophobic interactions between nucleoside bases and side chains of hydrophobic amino acids, rather than electrostatic forces, play a significant role in the ASL^{Phe} -peptide interaction. Aromatic amino acid residues

constitute about 50% of peptide t^{F2}'s sequence. The presence of only one charged amino acid residue in the peptide, Arg-12, also supports the hypothesis that the ASL^{Phe}–peptide interaction relies primarily on nonelectrostatic forces to recognize structural, modified nucleoside-dependent determinants in the ASL^{Phe}.

Recognition by hydrophobic interaction would not be unique to t^{F2}'s binding the anticodon of ASL^{Phe}. The interaction is consistent with that found in the X-ray crystallographic structure of lysyl-tRNA synthetase with cognate tRNA^{Lys} wherein a phenylalanine intercalates between anticodon nucleosides U₃₅ and U₃₆ (50). The peptide t^{F2} amino acid sequence has little in common with such RNA-binding proteins that are rich in arginine or lysine (51–53) or have zinc finger motifs (54, 55). There is no sequence homology with a zinc-dependent tRNA-binding peptide sequence from an aminoacyl-tRNA synthetase (56). Though peptide t^{F2} binds an RNA stem and loop domain, it has no sequence homology to stem and loop and single-stranded RNA-binding proteins (57–61) nor to double-stranded RNA-binding proteins (62). However, there are four serine residues within the 15 amino acid long sequence of peptide t^{F2}. The exclusive selection of serine among all the polar amino acids and its role in the ASL–peptide interaction is unknown so far, but it suggests the possibility that hydrogen bonds formed by serines may be important for the interaction. All three RPL selected peptides had one serine in the C-terminal half of their sequences. However, the number of serines in the other two RPL-selected peptides, t^{F3} and t^{F33}, was reduced.

Peptide t^{F2} has tRNA^{Phe} recognition properties and secondary structural characteristics in common with native phenylalanyl-tRNA synthetases (FRS). The presence of a serine along with an arginine and a tyrosine toward the C-terminal half of all three peptides may be important for binding the ASL and tRNA. Five nucleosides in single-stranded regions of yeast tRNA^{Phe} including those of the anticodon are identity elements crucial to the tRNAs recognition by yeast FRS (13, 38). Yeast FRS preferentially aminoacylates the modified tRNA rather than the unmodified transcript (13, 38). Unfortunately, a structure of yeast tRNA^{Phe} with its cognate heterodimer FRS has not been reported. To date, the only reported high-resolution structure of a tRNA^{Phe} and FRS is that of *Thermus thermophilus* tRNA^{Phe} cocrystallized with its cognate FRS (63). The first nucleoside G₃₄ of the GAA anticodon triplet is an important recognition determinant for both the yeast and *T. thermophilus* FRSs (13, 38, 63). In the cocrystal of *T. thermophilus* FRS with tRNA^{Phe}, four C-terminal amino acids, Arg, Ser, Asp, and Tyr, of the protein's anticodon-binding domain interact with G₃₄ (63). Three of the four amino acids, Arg, Ser, and Tyr, are present in peptides t^{F2}, t^{F3}, and t^{F33}, though the sequence of the tightest binding peptide t^{F2} has them in closest proximity at the C-terminus (Table 1). Peptide t^{F2} also has a β -structure in common with the C-terminal sequence of the *T. thermophilus* FRS anticodon recognition domain in the crystal. However, the degree to which β -structure is important to binding affinity is not obvious and may become evident with the structure determination of the t^{F2}–ASL^{Phe}–Cm₃₂,Gm₃₄,m⁵C₄₀ complex.

Peptide t^{F2}'s preferential recognition of the properties contributed by Gm₃₄ in the loop and m⁵C₄₀ on the 3'-side of the stem and the FRET from peptide t^{F2} to wybutosine in

the native tRNA^{Phe} indicate that the peptide may be located across the anticodon and onto the 3'-side of the stem. The peptide interaction with tRNA^{Phe} may be similar to that of FRS with tRNA^{Phe}. Because peptide t^{F2} and yeast FRS have similar K_d 's for tRNA^{Phe} and the peptide has proximal Arg, Ser, and Tyr that are critical to *T. thermophilus* FRS recognition of tRNA^{Phe} G₃₄, it is not surprising that peptide t^{F2} inhibited FRS aminoacylation of yeast tRNA^{Phe}. In fact, the ratio of experimentally determined initial to inhibited rates of aminoacylation, 2.0, was comparable to that calculated, 1.3, from the FRS K_m (13) and the K_d of t^{F2} for the tRNA; i.e.

$$v_0/v_i \approx 1 + (K_m/(K_m + [\text{tRNA}^{\text{Phe}}]))([\text{t}^{\text{F2}}]/K_d)$$

where [tRNA^{Phe}] and [t^{F2}] are the tRNA and peptide concentrations, respectively.

The ability of t^{F2} to inhibit FRS and its having amino acid residues in common to that of the *T. thermophilus* FRS anticodon binding domain demonstrates that RPL-selected peptides will provide insight into the contributions of modifications to RNA–protein interaction. The methylation-dependent binding of peptide t^{F2} to ASL^{Phe} demonstrates the importance of one of the simplest of modifications to contribute significantly to RNA identity and to protein recognition. Whether t^{F2} is recognizing the methyl groups directly, or the structure they impart to the RNA, will become evident from our determination of the structure of the complex in solution using NMR spectroscopy.

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SUPPORTING INFORMATION AVAILABLE

Three figures showing the migration of ASL^{Phe}–Cm₃₂,Gm₃₄,m⁵C₄₀ in native gel electrophoresis, stoichiometry of ASL^{Phe}–Cm₃₂,Gm₃₄,m⁵C₄₀ binding by peptide t^{F2}, and ribosome-mediated codon binding of ASL^{Phe}–Cm₃₂,Gm₃₄,m⁵C₄₀ not inhibited by peptide t^{F2}. This material is available free of charge via the Internet at <http://pubs.acs.org>.

REFERENCES

- Mattaj, I. W., and Nagai, K. (1995) *Nat. Struct. Biol.* 2, 518–522.
- Ashraf, S. S., Sochacka, E., Cain, R., Guenther, R., Malkiewicz, A., and Agris, P. F. (1999) *RNA* 5, 188–194.
- Yarian, C., Marszalak, M., Sochacka, E., Malkiewicz, A., Guenther, R., Miskiewicz, A., and Agris, P. F. (2000) *Biochemistry* 39, 13390–13395.
- Davis, D. (1998) in *Modification and Editing of RNA* (Grosjean, H., and Benne, R., Eds.) pp 85–102, American Society for Microbiology, Washington, DC.

5. Curran, J. F. (1998) in *Modification and Editing of RNA* (Grosjean, H., and Benne, R., Eds) pp 493–516, American Society for Microbiology, Washington, DC.
6. Bjork, G. R. (1995) in *tRNA: Structure, biosynthesis and function* (Söll, D., and RajBhandary, U. L., Eds.) pp165–205, American Society for Microbiology, Washington, DC.
7. Agris, P. F. (1996) *Prog. Nucleic Acids Res. Mol. Biol.* 5, 79–129.
8. Raychaudhuri, S., Conrad, J., Hall, B. G., and Ofengand, J. (1998) *RNA* 4, 1407–1417.
9. Helm, M., Giege, R., and Florentz, C. (1999) *Biochemistry* 38, 13338–13346.
10. Auxlien, S., Keith, G., Le Grice, S. F., and Darlix, J. L. (1999) *J. Biol. Chem.* 274, 4412–4420.
11. Madore, E., Florentz, C., Geige, R., Sekine, S., Yokoyama, S., and Lapointe, J. (1999) *Eur. J. Biochem.* 266, 1128–1135.
12. Freist, W., Gauss, D. H., Söll, D., and Lapointe, J. (1997) *J. Biol. Chem.* 272, 1313–1329.
13. Sampson, J. R., Behlen, L. S., DiRenzo, A. B., and Uhlenbeck, O. C. (1992) *Biochemistry* 31, 4161–4167.
14. Nazarenko, I. A., Harrington, K. M., and Uhlenbeck, O. C. (1994) *EMBO J.* 13, 2464–2471.
15. Putz, J., Florentz, C., Benseler, F., and Giege, R. (1994) *Nat. Struct. Biol.* 1, 580–582.
16. Muramatsu, T., Nishikawa, K., Nemoto, F., Kuchino, Y., Nishimura, S., Miyazawa, T., and Yokoyama, S. (1988) *Nature* 336, 179–181.
17. Yarian, C. S., Cain, R., Basti, M. M., Ansari, G., Guenther, R. H., Sochacka, E., Malkiewicz, A., and Agris, P. F. (1999) *Nucleic Acids Res.* 27, 3543–3549.
18. Sundaram, M., Durant, P. C., and Davis, D. R. (2000) *Biochemistry* 39, 12575–12584.
19. Stuart, J. W., Gdaniec, Z., Guenther, R., Marszalek, M., Sochacka, E., Malkiewicz, A., and Agris, P. F. (2000) *Biochemistry* 39, 13396–13404.
20. Koshlap, K. M., Guenther, R. H., Sochacka, E., Malkiewicz, A., and Agris, P. F. (1999) *Biochemistry* 38, 8647–8656.
21. Sengupta, R., Vainauskas, S., Yarian, C., Sochacka, E., Malkiewicz, A., Guenther, R. H., Koshlap, K. M., and Agris, P. F. (2000) *Nucleic Acids Res.* 28, 1374–1380.
22. Auld, D. S., and Schimmel, P. (1995) *Science* 267, 1994–1996.
23. Auld, D. S., and Schimmel, P. (1998) *EMBO J.* 15, 1142–1148.
24. Agris, P. F., Marchbank, M. T., Newman, W., Guenther, R., Ingram, P., Swallow, J., Mucha, P., Szyk, A., Rekowski, P., Peletskaya, E., and Deutscher, S. L. (1999) *J. Protein Chem.* 18, 425–435.
25. Ashraf, S. S., Guenther, R. H., Ansari, G., Malkiewicz, A., Sochacka, E., and Agris, P. F. (2000) *Cell Biochem. Biophys.* 33, 241–252.
26. Dao, V., Guenther, R., Malkiewicz, A., Nawrot, B., Sochacka, E., Kraszewski, A., Jankowska, J., Everett, K., and Agris, P. F. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 2125–2129.
27. Chen, Y., Sierzputowska-Gracz, H., Guenther, R., Everett, K., and Agris, P. F. (1993) *Biochemistry* 32, 10249–10253.
28. Ashraf, S. S., Ansari, H., Guenther, G., Sochacka, E., Malkiewicz, A., and Agris, P. F. (1999) *RNA* 5, 503–511.
29. Agris, P. F., Malkiewicz, A., Kraszewski, A., Everett, K., Nawrot, B., Sochacka, E., Jankowska, J., and Guenther, R. (1995) *Biochimie* 77, 125–134.
30. Beaucage, S. L., and Caruthers, M. H. (2000) in *Current Protocols in Nucleic Acid Chemistry* (Beaucage, S. L., Bergstrom, D. E., Glick, D. G., and Jones, R. A., Eds.) pp 3.3.1–3.3.20, John Wiley & Sons, New York.
31. Wolfson, A. D., Pleiss, J. A., and Uhlenbeck, O. C. (1998) *RNA* 4, 1019–1023.
32. Mucha, P., Rekowski, P., Szyk, A., Newman, W., Guenther, R., Ingram, P., Swallow, J., Agris, P. F., Marchbank, M. T., Deutscher, S. L., and Peletskaya, E. (1998) *Peptides*, 608–609.
33. Sprinzl, M., Horn, C., Brown, M., Ioudovitch, A., and Steinberg, S. (1998) *Nucleic Acids Res.* 26, 148–153.
34. Smith, C., Schmidt, P. G., Petsch, J., and Agris, P. F. (1985) *Biochemistry* 24, 1434–1440.
35. Turner, D. H., Tinoco, I., Jr., and Maestre, M. F. (1975) *Biochemistry* 14, 3794–3799.
36. Nagamatsu, K. (1989) *J. Biomol. Struct. Dyn.* 6, 729–739.
37. Lahiri, A., and Nilsson, L. (2000) *Biophys. J.* 79, 2276–2289.
38. Sampson, J. R., DiRenzo, A. B., Behlen, L. S., and Uhlenbeck, O. C. (1990) *Biochemistry* 29, 2523–2532.
39. Sampson, J. R., DiRenzo, A. B., Behlen, L. S., and Uhlenbeck, O. C. (1989) *Science* 243, 1363–1366.
40. Pozdnyakova, I., Guidry, J., and Witung-Stafshede, A. (2000) *J. Am. Chem. Soc.* 122, 6337–6338.
41. Munoz, V., Thompson, P. A., Hofrichter, J., and Eaton, W. A. (1997) *Nature* 390, 196–199.
42. Wright, P. E., Dyson, H. J., and Lerner, R. A. (1988) *Biochemistry* 27, 7167–7175.
43. Manavalan, P., and Johnson, W. C., Jr. (1987) *Anal. Biochem.* 167, 76–85.
44. Dalmas, B., Hunter, G. J., and Bannister, W. H. (1994) *Biochem. Mol. Biol. Int.* 34, 17–26.
45. Reed, J., and Reed, T. A. (1997) *Anal. Biochem.* 254, 36–40.
46. Bjork, G. R., Durand, J. M. B., Hagervall, T. G., Leipuviene, R., Lundgren, H. K., Nilsson, K., Chen, P., Quian, Q., and Urbonavicius, J. (1999) *FEBS Lett.* 452, 47–51.
47. Basti, M. M., Stuart, J. W., Lam, A. T., Guenther, R., and Agris, P. F. (1996) *Nat. Struct. Biol.* 3, 38–44.
48. McClain, W. H., and Seidman, J. G. (1975) *Nature* 257, 106–110.
49. Sirum-Connolly, K., and Mason, T. L. (1993) *Science* 262, 1886–1892.
50. Cusack, S., Yaremchuk, A., and Tukalo, M. (1996) *EMBO J.* 15, 6321–6334.
51. Long, K. S., and Crothers, D. M. (1995) *Biochemistry* 34, 8885–8895.
52. Hemmerich, P., Bosbach, S., von Mikecz, A., and Krawinkel, U. (1997) *Eur. J. Biochem.* 245, 549–556.
53. Gosser, Y., Hermann, T., Majumdar, A., Hu, W., Frederick, R., Jiang, F., Xu, W., and Patel, D. J. (2001) *Nat. Struct. Biol.* 8, 146–150.
54. Amarasinghe, G. K., De Guzman, R. N., Turner, R. B., Chancellor, K. J., Wu, Z. R., and Summers, M. F. (2000) *J. Mol. Biol.* 301, 491–511.
55. Friesen, W. J., and Darby, M. K. (2001) *J. Biol. Chem.* 276, 1968–1973.
56. Glasfeld, E., and Schimmel, P. (1997) *Biochemistry* 36, 6739–6744.
57. Meehan, H. A., and Connell, G. J. (2001) *J. Biol. Chem.* (in press).
58. Dominski, Z., Erkmann, J. A., Greenland, J. A., and Marzluff, W. F. (2001) *Mol. Cell. Biol.* 21, 2008–2017.
59. Battle, D. J., and Doudna, J. A. (2001) *RNA* 7, 123–132.
60. Spangord, R. J., and Beal, P. A. (2001) *Biochemistry* 40, 4272–4280.
61. Ginisty, H., Amalric, F., and Bouvet, P. (2001) *J. Biol. Chem.* 276, 14338–14343.
62. Fierro-Monti, I., and Mathews, M. B. (2000) *Trends Biochem. Sci.* 25, 241–246.
63. Goldrik, Y., Mosyak, L., Reshetnikova, L., Ankilova, V., Lavrik, O., Khodyreva, S., and Safro, M. (1997) *Structure* 5, 59–68.