Aminoacyl-tRNA Recognition by the Leucyl/Phenylalanyl-tRNA-Protein Transferase*

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We employ mutant and mischarged aminoacyl-tRNAs to characterize aminoacyl-tRNA recognition by the leucyl/phenylalanyl-tRNA-protein transferase (L/F-transferase). Wild type Met-tRNA

\[\text{Met}^{\text{f}}\text{tRNA}\] (CAU anticodon) and mischarged Met-tRNA

\[\text{Met}^{\text{m}}\text{tRNA}\] (UAA anticodon) are each 0.4 aminoacyl-tRNA hybrids. tRNA, deoxyoligonucleotide

\[\text{dO}\text{tRNA}\] transferase). Wild type Met-tRNA

\[\text{Met}^{\text{f}}\text{tRNA}\] (CAU anticodon) to characterize aminoacyl-tRNA recognition by the Leu-tRNALeu-4 fusion controlled. Finally, deoxyoligonucleotide

\[\text{L/F-transferase complex is slow, relative to the rate calculated that association is diffusion controlled.}

\[\text{dO}\text{tRNA}\] accepts for the L/F-transferase, and the dissociation constants of the complexes between L/F-transferase and either wild type Leu-tRNALeu-4 (UAA) or mutant Leu-tRNALeu-4 (CUA) are each 0.4 ± 0.2 μM. The dissociation constants for the complexes between the L/F-transferase and uncharged tRNA, leucine methyl ester, and puromycin are all 10–1,000-fold greater than that of the Leu-tRNAL/F-transferase complex. Dissociation of the Leu-tRNAL/F-transferase complex is slow, relative to the rate calculated that association is diffusion controlled. Finally, deoxyoligonucleotide

\[\text{dO}\text{tRNA}\] acceptor stem recognized by the L/F-transferase. A dOAA-tRNA completely lacking acceptor stem base pairs remains a substrate for the L/F-transferase, whereas a dOAA-tRNA containing a 2-base pair single-stranded region, at its 3′ terminus, does not.

The L/F-transferase catalyzes the transfer of Leu, Phe, and Met from aminoacyl-tRNAs to the amino termini of acceptor proteins (1, 2). All known acceptor proteins or peptides contain the basic amino-terminal residues Arg or Lys and are predominantly unstructured at their amino termini (3). The use of aminoacyl-tRNA as the aminoacyl donor is analogous to the enzymatic and lacks detectable RNA component or other organic cofactors (15, 16). The gene encoding the L/F-transferase has been cloned and used to overexpress, purify, and characterize the recombinant L/F-transferase homologs, lie at the end of three gene operons whose other members are homologs of the P-glycoproteins responsible for multi-drug resistance in mammalian cells (8, 12). These multi-drug resistance homologs are essential for exit from the stationary phase (13) and for the proper expression of cytochrome d (14) in E. coli cells. This chromosomal position remains an additional clue to the cellular function of the L/F-transferase.

The L/F-transferase is a monomeric enzyme of 234 residues that lacks a detectable RNA component or other organic cofactors (15, 16). The gene encoding the L/F-transferase has been cloned and used to overexpress, purify, and characterize the L/F-transferase. The specific activity of the recombinant L/F-transferase is comparable to that of the previously purified wild type enzyme (15). Circular dichroism analysis demonstrates that the L/F-transferase is ~50% a-helical and lacks detectable β-sheet structure. The absence of β-sheet structure strongly suggests that the L/F-transferase recognizes tRNA using a domain other than the ribonucleoprotein RNA-binding domain, found throughout the prokaryotic and eukaryotic kingdoms (17). Using the recombinant enzyme, we demonstrated previously that the modified nucleotides found in natural tRNAs are not essential for recognition by the L/F-transferase (16). This result opens the way for the use of T7 RNA polymerase-derived mutant tRNAs to dissect the essential determinants of aminoacyl-tRNAs recognized by the L/F-transferase.

MATERIALS AND METHODS

\[\text{tRNA and Aminoacyl-tRNA Synthesis and Purification—Unmodified tRNAs were transcribed using T7 RNA polymerase using standard procedures with minor modifications (18). Briefly, plasmid-borne tRNA genes were digested with BsrNI restriction endonuclease (New England Biolabs) and the enzyme removed by phenol/chloroform extraction.} \]
Transcription reactions included 40 mM Tris (pH 8.0), 20 mM MgCl₂, 25 mM KCl, 2.0 mM spermidine, 2.0 mM dithiothreitol, 16 mM each nucleotide triphosphate, 40 mM GMP, 80 units of RNAsin (Promega), and purified T7 RNA polymerase. Transcription reactions were incubated 8–10 h at 37 °C. Heterogeneous RNA transcripts were separated from unincorporated nucleotides and pyrophosphate using a NAP-5 column (Pharmacia) followed by precipitation with 2 volumes of ethanol in the presence of 0.2 mM NaCl. Full-length RNA transcripts were isolated from incorporated transcripts by electrophoresis on 10% acrylamide gels containing 50% urea. Full-length RNA transcripts were identified by their UV shadow, eluted from the gel slice by soaking overnight at 37 °C, and concentrated by ethanol precipitation. Leu-tRNAs were generated using partially purified E. coli Leu-tRNA synthetase (LeuRS) as described previously (16). All reactions contain 50 mM Tris (pH 7.2) and 1–2 mM ATP, 20 μM Leu. Aminoacyl-tRNAs were separated from the LeuRS by phenol/chloroform extraction using phenol equilibrated at pH 5.0. Aminoacyl-tRNAs were concentrated by precipitation with 2.5 volumes of ethanol in 0.2 mM NaCl.

L/F-Transferase Activity Assays—Qualitative reactions to characterize the ability of mischarged and mutant aminoacyl-tRNAs to serve as substrates for the L/F-transferase encode a mixture of aminoacyl-tRNA synthetases (Sigma) and purified L/F-transferase enzyme (16). All reactions contain 50 mM Tris (pH 8.0), 0.1 mM KCl, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 12 μM α-casein, 10 mM ATP, ~500 units of E. coli aminoacyl-tRNA synthetase (Sigma), and purified L/F-transferase. Reactions were incubated for 5 min at 37 °C. Reactions are assayed for [5-C]-casein (incorporated into protein and cold trichloroacetic acid stable) and [4-C]-amino acid incorporated aminoacyl-tRNA (cold trichloroacetic acid stable) as described previously (16).

Substrate Binding and Competitive Dissociation—Dissociation constants were determined using the ribonuclease A protection assay that is commonly employed to characterize aminoacyl-tRNA elongation factor Tu (AA-tRNAEF Tu) complex formation (19). Binding reactions are carried out in 50 mM Hepes buffer (pH 7.2) and contain 1–2 μM [3H]-Leu-tRNA_{leu} (CAG) or the amber suppressor [3H]-Leu-tRNA_{leu} (CAG) and variable amounts of purified L/F-transferase. Binding reactions are incubated for 15 min at 25 or 0 °C (on ice). The amount of aminoacyl-tRNA in complex with the L/F-transferase is determined by the addition of ribonuclease A to a final concentration of 0.25 mg/ml, followed by incubation on ice or at 25 °C for 15 s. Ribonuclease A digestion is stopped by the addition of equal volumes of 20% trichloroacetic acid, and 10-μl aliquots are spotted on Whatman 3MM filters. Filters are subsequently washed and the amount of radioactivity that is insoluble in trichloroacetic acid determined as described previously (16). In these experiments each value required to determine K_d is measured directly, K_d = ([tRNA/L-F-transferase]/[tRNA-L-F]). [tRNA-L-F] is determined from the number of counts of [3H]-Leu insoluble in 10% trichloroacetic acid after ribonuclease A digestion. Counts are converted to nanomoles, and concentrations, using the conversion factor 1 mmol of [3H]-Leu = 468,000 counts (empirically determined for the batch of [3H]-Leu used throughout this study). Leu-tRNA_{leu} is calculated from the number of counts of [3H]-Leu insoluble in 10% trichloroacetic acid before ribonuclease A digestion. [L/F-transferase] is determined, for each preparation, by reverse titration experiments by equating the amount of enzyme with the amount of [3H]-Leu-tRNA protected under saturating conditions. Competitive binding reactions are similar to those just described, except that the initial incubations are carried out in the presence of variable amounts of leucine methyl ester (Leu-O-Me, Bachem), puromycin (3'-[aminomethyl]-hydrocinnamamido)-3'-deoxy-N,N-dimethyladenosine, Sigma), or uncharged tRNA_{leu}.

Decoy oligonucleotide-Aminoacyl-tRNA Hybrid Formation—Hybridization of decynucleotides with aminoacyl-tRNAs was performed by mixing Leu-tRNA_{leu} (4–6 μM) with oligonucleotide (40–50 μM) and incubation for 30 s at 75–80 °C, followed by slow cooling to 20 °C. These hybrids are carried out in 10 mM sodium acetate (pH 5.0) to minimize spontaneous hydrolysis of aminoacyl-tRNAs. Hybridization reactions are subsequently precipitated with ethanol and stored at −75 °C. Immediately prior to use, hybrids are resuspended in H₂O.

One-half of the resuspended hybrid is used as substrate in the L/F-transferase reaction while the remainder is assayed for the fraction of Leu-tRNA_{leu} aminoacyl-tRNA in the hybrid form by non-denaturing gel electrophoresis in TAE buffer (40 mM Tris acetate, 10 mM EDTA). Decoy oligonucleotides used in hybrid formation were as follows: LP, 5'-ACCCGAGG-GGACCTTCAAC-3'; LCA, 5'-TCGGGAGGAGGGCTGAAC-3'; L2, 5'-TGACCCGAGGAGGGCTGAAC-3'; L30, 5'-CTACCCGATCACA-CC ATCGGG-3'.

RESULTS

Mischarged Aminoacyl-tRNAs in the L/F-Transferase Reaction—The abilities of selected aminoacyl-tRNAs and aminoacyl-tRNA mutants to function as substrates of the L/F-transferase during the NH₂-terminal aminoacylation of α-casein are shown in Table I (upper section). Wild type tRNA_{Met} (CAU) and the anticodon mutant tRNA_{Val} (CAU) are each aminoacylated with methionine to form Met-tRNA_{Met} (CAU) and Met-tRNA_{Val} (CAU), respectively. These two Met-tRNAs serve as substrates for the L/F-transferase in the NH₂-terminal aminoacylation of α-casein (Met-tRNA_{Val} (CAU) contains the nucleotide sequence of tRNA_{Val} but with a CAU anticodon; the anticodon of wild type methionine tRNAs). Conversely, Val is not transferred from either its wild type cognate Val-tRNA_{Val} (UAC) or from the anticodon mutant Val-tRNA_{Met} (UAC), and Arg is not transferred from Arg-tRNA_{Met} (CCG, A20). These results suggest that either the aminoacyl moiety of an aminoacyl-tRNA is important for recognition by the L/F-transferase, or that the L/F-transferase recognizes directly the anticodons of its aminoacyl-tRNA substrates (this second model is not supported by additional experiments, described below). Each of the amino acids transferred by the L/F-transferase are hydrophobic and contain an unbranched β-carbon. The lack of transfer of either Val (hydrophobic) or Arg (unbranched β-carbon) from tRNAs that are nearly identical with tRNA_{Met}, suggests that both properties of the amino acid moiety are essential for recognition by the L/F-transferase. The experiments described above rely on the recognition specificities of specific aminoacyl-tRNA synthetases. For example, the Met- and Val-tRNA synthetases from E. coli identify their cognate tRNAs predominantly via direct recognition of the nucleotides within the tRNA anticodon (20). Other nucleotides in the tRNA structure may be mutated with minor effect. Correspondingly a tRNA_{Met} with the Val anticodon (UAC) is efficiently charged with Val but not Met, while tRNA_{Val} with the Met anticodon (CAU) is efficiently charged with Met but not Val. In addition to the Arg anticodon (CCG), a second mutation, U20 → A20, is required to convert tRNA_{Met} into an efficient substrate for the Arg-tRNA synthetase (21).

Effects of Mutations in the Anticodon and Extra Arm of tRNA<sup>α</sup> in the L/F-Transferase Reaction—The specificities of the L/F-transferase for specific aminoacyl-tRNA mutants (Table I) left open the possibility that the L/F-transferase might recognize the anticodon of its cognate aminoacyl-tRNAs. This mode of recognition has been demonstrated for several aminoacyl synthetases in addition to those mentioned above (20). Also, the base of the central nucleotide of the anticodon of all L/F-transferase-substrate aminoacyl-tRNAs is an adenine. Central adenines in the tRNA anticodons are required to recognize Leu codons (CUN and UUR), Phe codons (UUY), and the Met codon (AUG). This common adenine provides for a plausible mechanism for the specific recognition of these three classes of tRNA by a single enzyme. However, this mode of recognition is strongly argued against by the data in Table I (lower section). A tRNA<sub>Leu</sub>-1 anticodon mutant (UCA anticodon), a second mutant containing a G97 → U97 substitution in the anticodon loop (immediately 3' to the anticodon), and a third mutant tRNA<sub>Leu</sub>-2 lacking four nucleotides of the extra arm region (32 lacks the C47-G47g and C47a-G47h base pairs (22)), are all active as L/F-transferase substrates. These data demonstrate that the anticodon is not an essential determinant for the recognition of aminoacyl-tRNAs by the L/F-transferase. Nonetheless, experiments described below reveal that the nucleic acid makes a contribution to the overall affinity of the AA-tRNA/LF complex. It should be noted that the appearance of some of the experiments takes advantage of the fact that the majority of
Certain unmodified and mischarged AA-tRNAs are substrates for the L/F-transferase

Table I

| tRNA (anticodon) | Amino acid | Cold trichloroacetic acid | Hot trichloroacetic acid | R |
|------------------|------------|--------------------------|-------------------------|---|
| Metm (CAU)       | [14C]Met  | 10,300                   | 6,100                   | 60 |
| Val (CAU)        | [35S]Met  | 6,200                    | 2,100                   | 34 |
| Metm (UAC)       | [14C]Val  | ~0                       | ~0                      | ~0 |
| Val (UAC)        | [14C]Val  | 6,100                    | ~0                      | ~0 |
| Metm (CAU)       | [35S]Val  | 9,600                    | ~0                      | ~0 |
| Val (CAU)        | [35S]Val  | ~0                       | ~0                      | ~0 |
| Metm (CCG, A20)  | [35S]Arg  | 15,200                   | ~0                      | ~0 |
| Leu1             | [14C]Leu  | 7,700                    | 1,900                   | 24 |
| Leu1, Δ2         |            | 7,650                    | 1,700                   | 22 |
| Leu1, U37        |            | 7,450                    | 1,750                   | 23 |
| Leu1 (UGA)       |            | 7,400                    | 1,800                   | 24 |

The ability of a mutant tRNA to serve as a substrate for the L/F-transferase strongly depends on the methionyl moiety. Qualitative comparisons of the effects of mutations in tRNA<sup>Metm</sup> and tRNA<sup>Val1</sup> on their ability to serve as substrates for the L/F-transferase were made using coupled reactions containing purified L/F-transferase and a mixture of total E. coli aminoacyl-tRNA synthetases. Cold trichloroacetic acid is the amount of [14C]valine or [35S]methionine (in counts) that is precipitated by 10% trichloroacetic acid after the reaction and is indicative of radiolabeled amino acid incorporated into protein. R is the ratio: Hot trichloroacetic acid/Cold trichloroacetic acid (in percent). tRNA<sup>Metm</sup> (CAU) = 5'-GGCUAAGCUACGUGUUGGUAUGAGCAACUACUCCUAGAUAGGGGAGCAUGGCGUCCACC' is the E. coli wild type elongator tRNA<sup>Metm</sup> (anticodon underlined). tRNA<sup>Val1</sup> (UAC) = tRNA<sup>Metm</sup> (CAU) with a CAU→UAC anticodon mutation, tRNA<sup>Metm</sup> (CCG, A20) = tRNA<sup>Metm</sup> (CAU) with a CAU→CCG anticodon mutation and a T→A mutation at nucleotide 20 (lower case). tRNA<sup>Aux−1</sup> (UAC) = 5'-GGGUGAUAGCUACGUGGGAAGACGACGCUCUCAACAGGGGGGAGCAUGCUCGCCUCAUCCCAAGAGGCUAGGCCCAGGGUCGCUACACCCACCA-3' is one of the three wild type tRNA<sup>Aux−1</sup> s of E. coli, tRNA<sup>Aux−3</sup> (CAU) = tRNA<sup>Aux−1</sup> (UAC) with a UAC→CAU anticodon mutation.

Lower, mutations in neither the anticodon nor the extra arm abolish the ability of Leu-tRNA<sup>Leu−1</sup> mutants to function as L/F-transferase substrates. Qualitative comparisons of the effects of mutations in tRNA<sup>Leu−1</sup> on its activity as a substrate for the L/F-transferase were made using coupled reactions containing purified L/F-transferase and purified Leu-tRNA synthetase. tRNA<sup>Leu−1</sup> = tRNA<sup>leu−1</sup> GCAAAAGGGGGGAAUUGGUAAGCGCGGCUACGUGGUAAGGCUACGUGGUAAGCUCUACACAGAGGCUAGGCCCAGGGUCGCUACACCCACCA-3'. tRNA<sup>Leu−1</sup> = tRNA<sup>Leu−1</sup> but lacking 4 nucleotides from the extra arm region (shown in lower case), tRNA<sup>Leu−1</sup> U37 = tRNA<sup>Leu−1</sup> with a G→U mutation at nucleotide 37 (immediately 3' to the anticodon), tRNA<sup>Leu−1</sup> (UGA) = tRNA<sup>Leu−1</sup> with a CAG→UGA mutation in the anticodon.

The K<sub>d</sub> value is significantly lower than that of the L/F-transferase-Eucalyptus Fashion Tu complexes (2–10 nm) (19). This lower affinity of the L/F-transferase for aminoacyl-tRNAs is consistent with our earlier findings that the L/F-transferase can be overexpressed to high cellular levels without causing significant lethality (16).

**Competition for L/F-transferase Binding by Leucine Methyl Ester, Ester, and Puromycin**—To dissect further the regions of Leu-tRNA recognized by the L/F-transferase we carried out binding reactions in the presence of variable amounts of competitor molecules. The simplest competitor tested was the methyl ester of leucine (Leu-O-Me). Competition experiments employing the Leu-O-Me molecule allow estimation of the importance of the leucyl side chain and free amino group of Leu-tRNA for recognition by L/F-transferase. NH<sub>2</sub> terminal acetylated Phe-tRNA<sup>Phe</sup> is not a substrate for the L/F-transferase (24). The use of the ester removes the possible confusing contribution to the binding reaction of the charged acid group of free Leu, as it seems likely that the L/F-transferase uses discrimination against this charge to avoid binding free leucine in the cell. Leu-O-Me demonstrates a measurable ability to compete with Leu-tRNA for L/F-transferase binding (Fig. 2). Based on the competition data, the <i>K<sub>S</sub></i> of the Leu-O-Me/L/F complex is >1.0 mM, a value significantly higher than that of the tRNA<sup>Leu−1</sup>/L/F complex (see Fig. 1). This suggests that the nucleic acid moieties of aminoacyl-tRNAs contribute to their overall affinity for the L/F-transferase. Unfortunately, a quantitative determination of the ability of uncharged tRNA to bind the L/F-transferase and inhibit the formation of the AA-tRNA/L/F complex is not possible using the ribonuclease A protection assay. The concentrations of tRNA<sup>Leu−1</sup> required to compete significantly with Leu-tRNA<sup>Leu−1</sup> for limiting L/F-transferase overwhelm the ability of ribonuclease A to degrade completely the unbound Leu-tRNAs. However, an 8-fold excess of uncharged tRNA<sup>Leu−1</sup> over Leu-tRNA<sup>Leu−4</sup> shows no detectable effect on L/F-transferase-Leu-tRNA<sup>Leu−4</sup> complex formation (data not shown). This result demonstrates, at least qualita-
somewhat proteinsynthesisthatactsviaitsabilitytomimicthe3'-dimethyladenosine) is a widely used inhibitor of ribonucleases and cause polypeptide chain termination. Puromycin also inhibits aminoacylation of the aminoacyl-tRNA, is a significantly better inhibitor of the aminoacyl-tRNA hybrids that mimic both the aminoacyl and tRNA moieties of an aminoacyl-tRNA, is a wild type tRNA-Leu of E. coli, trNAa<sup>aa</sup>-t(UAG), trNA<sup>a</sup>-t(UUA) with a UUA → UAG anticodon mutation.

Puromycin (3′-[amino-p-methoxy-hydrocinnamamido]-3′-deoxy-N,N-dimethyladenosine) is a widely used inhibitor of ribosomal protein synthesis that acts via its ability to mimic the 3′-terminal adenine of aminoacylated tRNA and cause polypeptide chain termination. Puromycin also inhibits aminoacylation of α-casein by the L/F-transferase (25). As seen in Fig. 2, puromycin, which mimics both the aminoacyl and tRNA moieties of an aminoacyl-tRNA, is a significantly better inhibitor of the L/F-transferase than Leu-O-Me. This result supports the claim that the tRNA moiety of aminoacyl-tRNAs make a contribution to the overall affinity of the L/F-transferase. In order to determine the importance of the different structural elements contained within the tRNA acceptor stem, the structure of this region was modulated by constructing deoxyoligonucleotide hybrids (dOligoAA-tRNAs). This approach was necessitated by the inability of the Leu-, Phe-, or Met-tRNA synthetases to charge efficiently a tRNA minihelix or microhelix (28). Because the hybrids are not expected to be aminoacylated efficiently by the LeuRS enzyme, the oligonucleotides were hybridized with previously aminoacylated tRNA<sub>Leu</sub>-4. The dO-AA-tRNAs are schematized in Fig. 4A and the completeness of hybrid formation, for each oligonucleotide, is revealed by the non-denaturing gel in Fig. 4b. Hybridization of the LP deoxyoligonucleotide recreates an anticodon helix that is structurally analogous to that seen in native aminoacyl-tRNAs, while use of the LCA oligonucleotide creates an acceptor helix with a terminal non-Watson-Crick T:U base pair. Oligonucleotide L2 generates an acceptor helix that exposes only a 2-base pair stranded 3′-terminus by hybridizing with the 5′ region of the tRNA. The data in Table II demonstrates that the dOligoAA-tRNAs constructed from deoxyoligonucleotides LP and LCA remain substrates for the L/F-transferase. Therefore, no 2′-OH

![Fig. 1. The dissociation constant of the aminoacyl-tRNA/L/F-transferase complex.](http://www.jbc.org/)

![Fig. 2. Formation of the AA-tRNA/L/F complex in the presence of inhibitor molecules.](http://www.jbc.org/)
groups from the 5'-strand are required for nucleic acid recognition by the L/F-transferase. In addition, the dO-tRNA constructed from L30 serves as an L/F-transferase substrate while the dO-tRNA constructed from L2 does not. Overall, these experiments reveal that no double stranded structure is required for recognition by the L/F-transferase, but rather that >2 single stranded nucleotides must be exposed at the 3' terminus of the aminoacyl-tRNA.

**DISCUSSION**

The L/F-transferase utilizes a highly degenerate family of macromolecules as substrates during its catalysis of peptide bond formation. For example, acceptor proteins and peptides bearing either of the basic residues Arg or Lys at their NH$_2$ terminus are efficient amino acid acceptors (3). In addition, Leu, Phe, Met, and the amino acid analog p-fluoro-Phe are all transferred from their cognate tRNAs, and certain mutant tRNAs, to acceptor proteins (24). The molecular bases for the degenerate recognition of aminoacyl-tRNAs by the L/F-transferase is becoming clear. Both the aminoacyl and nucleic acid moieties of an aminoacyl-tRNA are utilized by the enzyme during this discrimination. Previous authors have demonstrated that Phe-tRNA$_{Val-1}$ is a substrate but Val-tRNA$_{Val-1}$ and N-acetyl-Phe-tRNA$_{Phen}$ are not (24). We have generalized and extended these findings by demonstrating that Met-tRNA recognition is also strongly dependent on the aminoacyl group. The substrate specificity of the L/F-transferase for Leu-, Phe-, and Met-tRNAs, but not Ile- or Val-tRNAs suggests that an unbranched β-carbon is recognized by the enzyme. However, the inability of the enzyme to accept Arg-tRNA$_{Metm}$ (CCG, A20) as a substrate suggest that overall side chain hydrophobicity is also recognized.

Neither mutations in the anticodon region nor in the extra arm of tRNA$_{Leu-1}$ abolish recognition of this nucleic acid by the L/F-transferase. Direct recognition of the anticodon is observed for several aminoacyl-tRNA synthetases (20), however, the size of the L/F-transferase argued against its use of this mechanism. A globular protein of 234 amino acids would have an approximate diameter of 40 Å, while the distance from the anticodon loop to the aminoacyl group of an aminoacyl-tRNA is ~75 Å (29). Overall, the small size of the L/F-transferase and the lower $K_d$ of the puromycin-L/F complex, relative to Leu-O-Me-L/F complex, suggests that the nucleic acid residues recognized by the enzyme are very close to the 3' terminus of the tRNA. We demonstrated that no intramolecular base pairs involving the nucleotides from the 5' terminus of a tRNA are essential for recognition by the L/F-transferase. This is informative in light of the previous demonstration that the 3'-pen-

**Fig. 3.** Dissociation of the AA-tRNA/L/F complex is slow for each substrate/enzyme ratio tested. Qualitative estimation of the half-life of the tRNA/L/F complex is made using prolonged digestion of the complex with ribonuclease A. At each Leu-tRNA$_{Leu-4}$ (UUA)/L/F-transferase ratio, used to construct the dissociation curve of Fig. 2, the complex is stable for >45 s (left panel). For the binding reaction resulting in ~60% protection of tRNA$_{Leu-4}$ (UUA) a significant fraction of the complex is stable for >10 min (right panel).

**Fig. 4.** A, schematic of the dO-AA-tRNA hybrids. dO-tRNAs are constructed by annealing each deoxyoligonucleotide with previously aminoacylated tRNA$_{Leu-1}$ (UUA). The nucleotide sequence of tRNA$_{Leu-4}$ (UUA) is given in the legend to Fig. 1, the sequences of the deoxyoligonucleotides are: LP, 5'-ACCAGGAGGGAGCTCAAC-3'; LCA, 5'-TCGCCAGGCGGGAGCTCAGAC-3' (the nucleotide involved in the PU base pair is underlined); L2, 5'-GATCCGGAGCGGGGACTGAC-3'; L30, 5'-GCACGGATCCGACCCGAGGTAC-3'. B, a large fraction of each Leu-tRNA is converted to dO-Leu-tRNA hybrid form. Nondenaturing gel electrophoresis was used to demonstrate that the majority of the tRNA$_{Leu-4}$ (UUA) is converted to the dO-tRNA form in the annealing reactions. dO-Leu-tRNAs are expected to be less compact than fully folded tRNAs and therefore to have lower mobility during electrophoresis.

tanucleotide of Phe-tRNA$_{Phen}$ is not a substrate for the L/F-transferase (24). We conclude that nucleotides from the 3' terminus of an aminoacyl-tRNA 5' to the single-stranded 3'-terminal rA-rC-rC-rA are recognized by the L/F-transferase (not necessarily in base paired form). The existing data reflecting the ability of Phe-, Leu-, and Met-tRNAs to serve as L/F-transferase substrates are schematized in Fig. 5. The acceptor stem and anticodon of each tRNA is shown along with published data from L/F-transferase assays. The reported $K_a$ values for the different Leu isoacceptors vary over a nearly 10-fold range (Fig. 5) (23). A strong dependence on the nucleic acid moiety is also seen when the published data for the initiator and elongator Met-tRNA$_{Metm}$ are compared. These changes to the nucleic acid moiety result in a
Activity of dO-L-tRNAs hybrids as substrates for the L/F-transferase

| Oligonucleotide | Cold trichloroacetic acid | Hot trichloroacetic acid | \( R_1 \) | \( R_2 \) |
|-----------------|--------------------------|--------------------------|---------|---------|
| L2              | 750                      | 20                       | 0.03    | 0.04    |
| LCA             | 750                      | 550                      | 0.73    | 0.97    |
| None            | 1200                     | 900                      | 0.75    | 1.0     |
| LP              | 650                      | 500                      | 0.77    | 1.03    |
| L30             | 700                      | 450                      | 0.64    | 0.86    |

AA-tRNA-L/F-Transferase Interactions

During prolonged incubation in the presence of ribonuclease A, the observed \( t_{\text{off}} \) for dissociation of the L-tRNA-L/F complex is \( \sim 10 \text{ min} \) (see Fig. 3, right panel). If this process is equated to the dissolution of the ternary complex, the corresponding \( k_{-1} \) value is \( 1.1 \times 10^{-3} \text{ s}^{-1} \). Using the measured value: \( K_d = 0.4 \times 10^{-6} \text{ M} \), the calculated \( k_1 \) value for this process is then \( 2.750 \text{ s}^{-1} \), a value much lower than that expected for a diffusion controlled reaction (31). For the standard L/F-transferase reaction, as described by Soffer and colleagues (2), a 75-\( \mu \text{L} \) reaction containing 1.8 \( \mu \text{M} \) L-tRNA and 4.0 \( \mu \text{M} \) \( \alpha \)-casein are used. This concentration of \( \alpha \)-casein is \( \sim 4 \text{ times greater than the } K_a \) value for this substrate (1.0 \( \mu \text{M} \)), allowing the reaction to be approximated as pseudo-first order. Under these conditions in a reaction containing 1.0 \( \mu \text{g} \) L/F-transferase, formation of the ternary complex would occur at a calculated initial rate of: \( d[\text{ternary complex}] / dt = k_{2} \frac{[\text{L/F}][\text{AA-tRNA}]}{K_{d}} = 0.23 \times 10^{-8} \text{ M s}^{-1} \). However, in actual L/F-transferase assays, performed under these conditions, the rate of product formation is \( 5.3 \times 10^{-8} \text{ M s}^{-1} \) (16). Therefore, the observed rate of product formation is significantly greater than the calculated maximum. This paradox is further exaggerated when the near equality of our measured \( K_d (0.4 \mu \text{M}, \text{Fig. 1}) \) and the previously reported value for \( K_m (0.32 \mu \text{M} \text{ for tRNA}^{\text{Met}} \text{euc-tRNA}^{\text{Leu-4}} (23)) \) is considered. The near equality of \( K_d \) and \( K_m \) for Equation 2 results from the condition \( k_{-1} \gg k_2 \). Under these conditions, if the true \( k_{-1} = k_{\text{off}} = 1.1 \times 10^{-3} \text{ s}^{-1} \), product formation would be immeasurably slow; a result not observed.

The physical basis for the unusual binding kinetics of the AA-tRNA-L/F complex remain to be determined. Our working model is that the AA-tRNA-L/F complex is able to isomerize into non-productive conformations that are unable to proceed to product but remain inaccessible to ribonuclease. It should be noted that in this scenario, the \( K_d \) we report here is the value that is relevant to discussions of the fraction of cellular aminoacyl-tRNAs complexed with L/F-transferase. Two lines of reasoning have led us to adopt our model. First, although one form of the AA-tRNA-EF-Tu complex is very long-lived (the low
temperature complex), this stable AA-tRNA-EF-Tu complex displays a 1:1 stoichiometry. Conversely, the physiologically relevant complex at higher temperature displays a 1:2 stoichiometry, and is much less long-lived (27). Second, non-productive binding modes are most easily envisioned for an enzyme with degenerate substrate binding specificities, and aminoacyl-tRNA recognition by the L/F-transferase is unusually degenerate. For example, if the L/F-transferase’s substrate binding site recognizes the charged polyphosphates of single-stranded regions of the tRNA in conjunction with a hydrophobic side chain, these interactions might be provided by multiple conformations of the AA-tRNA-L/F complex.

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