Carbodiimide modification analysis of aminoacylated yeast phenylalanine tRNA: evidence for change in the apex region

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ABSTRACT

The G- and U-specific reagent, carbodiimide was used to probe the solution structure of aminoacylated yeast phenylalanine tRNA. Both quantitative and qualitative changes in modification were observed when the modification patterns of tRNA-CCA(3'OH), tRNA-CCA(3'NH₂) and phe-tRNA-CCA(3'NH₂) were compared. Five nucleotides were modified in all cases, D16 and G20 in the D-loop, U33 and Gm34 in the anticodon loop and U47, in the region of the extra arm. Small changes occurred in the D-loop with incorporation of the adenosine analogue manifest as new, low levels of modification of G22 (D-stem) and a loss of sensitivity to Mg⁺² in modification of D16. Aminoacylation resulted in new modification of G19, modification of a residue in the TΨCG sequence, and a 2.5-fold increase in modification of G22. Taken together the results show that aminoacylation causes increased exposure of bases in the apex region of the L-shaped molecule where the D- and Ψ-loops are joined. The effects observed could occur as a consequence of stable or dynamic changes in conformation.

INTRODUCTION

Despite considerable attention the question of the effect of aminoacylation on tRNA structure has not yet been satisfactorily resolved. A broad assortment of physical and biochemical methods sensitive to many different features of tRNA structure have been used to study this phenomenon. On first inspection the results of the thirty or so studies described to date appear to be inconclusive. These results are nearly evenly divided between showing that aminoacylation has no detectable effect and demonstrating that readily observable, albeit often small changes do occur. Further scrutiny reveals considerably more consistency however, than is first apparent. Techniques which are primarily sensitive to gross alterations in secondary or tertiary structure have generally failed to detect an effect while methods capable of sensing more subtle and, in cases, local perturbations have often been successful (see ref. 1 for brief review). Taken together, these results suggest that aminoacylation does not effect a large, stable rearrangement of the structure, but

that less profound tertiary-level effects can take place. Results from several studies indicate that tRNA assumes a more open, extended form on acylation (1-3) and others cite evidence of specific changes in the apex region of the L-shaped tertiary structure (4,5), the dihydrouridine (D) and pseudouridine (Ψ) loops (6-8) and, not surprisingly, at the 3'-CCA_{OH} terminus (9,10); the anticodon region has never been implicated in any of these studies.

In addition to the strong likelihood that some of the negative results described stem from the use of techniques insensitive to changes that do occur, it is also likely that some of the apparent inconsistencies occur as a consequence of the assorted solution conditions used. That solution conditions are important and, indeed, can be critical was underscored by results from a recent study from our laboratory (1,11,12). Here, aminoacylation of yeast tRNA^{Phe} was observed to result in an 18% decrease in the translational diffusion coefficient and a greater net negative charge, but only at certain conditions of magnesium. The full effect was observed at Mg⁺² levels above 5 mM but not at all at concentrations below 1 mM--all at pH 7.2, 20°C, and ionic strength of 0.1 M.

Unfortunately, our knowledge of the acylation dependent effects that have been documented is still only slight and rather unspecific owing to a paucity of precise, direct evidence. Here, we describe results of a chemical modification analysis of aminoacyl-tRNA undertaken with a view to obtaining additional direct evidence of an effect and to gain insight into its structural basis. The tRNA selected was yeast phenylalanine tRNA owing to our extensive knowledge of its tertiary structure and because of existing evidence of acylation-dependent changes (1,4,6-9,13). Of added interest was the possibility of obtaining information about the magnesium dependent change in diffusivity alluded to above.

The chemical probe was 1-cyclohexyl-3(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate, commonly referred to simply as carbodiimide. This reagent reacts with guanine and uracil bases and the commonly occurring derivatives of these residues found in tRNA^{Phe}, specifically, dihydrouridine, pseudouridine, ribothymidine and methylguanine. Together, these modifiable residues make up more than 50% of all the bases in tRNA^{Phe} and include virtually every secondary and tertiary pairing in the molecule. As is the case with nearly all base modifying reagents carbodiimide reacts only with unpaired bases exposed to solvent. This particular probe has the advantages of being reactive at near-physiological solution conditions and at concentra-

tions that contribute little to the overall ionic strength. The reagent has been used previously with non-acylated yeast tRNA^{Phe} to characterize thermal unfolding (14) and ion-dependent changes in diffusion behavior (Fritzinger and Fournier, in preparation). In those studies and here carbodiimide was reacted with uniformly labeled [³²P]-tRNA^{Phe} and the sites and extents of reaction ascertained by two-dimensional oligonucleotide fingerprinting and subsequent base analyses.

Under the conditions for modification (pH 8, 25°C), the half-time of deacylation for phe-tRNA^{Phe} is about 65 minutes (unpublished results). Because 24 to 48 hour reaction times are required for good sensitivity, corresponding to greater than 50% modification for highly exposed residues and at least 5-10% for less reactive nucleotides (Fritzinger and Fournier, in preparation) it is clear that the tRNA would become deacylated well before these levels of modification could occur. For this reason, it was necessary to stabilize the aminoacyl linkage. Several methods have been devised for this purpose. Two of these involve modifying the amino acid moiety of previously acylated tRNA by either removing the α amino group with nitrous acid treatment (15) or N-blocking of the α amino group, usually by acetylation (16). Although both methods increase the stability of the linkage at least ten-fold neither was adopted for the present work. The deamination method is quite harsh and can result in some degradation of the tRNA itself (evidenced in the current study by RPC-5 chromatography-unpublished results). Further, both methods modify the amino group of the amino acid which could have the effect of mitigating acylation-dependent changes. Finally, although either treatment extends the half-time of deacylation by an order of magnitude, this increase may not be sufficient for the levels of modification desired.

The method chosen for stabilizing the aminoacyl-tRNA linkage in this study was that described first by Fraser and Rich (17) in which the 3' terminal adenosine is replaced with the analogue, 3'-deoxy-3'-aminoadenosine. Aminoacylation then results in the formation of an amide linkage between the amino acid and the tRNA that is completely resistant to hydrolysis under slightly basic conditions. The method for the preparation of the analogue-containing tRNA^{Phe} is shown in Figure 1 and described in detail in Methods.

One of the aims of this study was to examine the effect of magnesium on the structure of phe-tRNA. Our earlier light scattering work had shown that aminoacylation caused a marked decrease in the diffusivity of yeast tRNA^{Phe} in 10 mM MgCl₂ but no effect in 1 mM MgCl₂ (1,11,12). To gain additional insight into this phenomenon, the carbodiimide modification patterns of acylated and

non-acylated tRNA^{Phe} were examined in both 1 and 10 mM magnesium. The sites and extents of modification were determined by fingerprint and oligonucleotide analysis according to the strategy outlined in Figure 1 and described in Methods. A preliminary account of this work has been presented (18).

METHODS

Preparation of [³²P]-tRNA^{Phe}--[³²P]tRNA^{Phe} was purified by a new two-step procedure to be described in detail elsewhere (Fritzinger and Fournier, in preparation). Briefly, the method involves isolation of *in vivo* labeled bulk tRNA by conventional means and fractionation by benzoylated DEAE cellulose chromatography and two-dimensional electrophoresis in gels of polyacrylamide. Starting with a 250 ml culture labeled with 50 mCi [³²P]orthophosphate, the procedure generally yields 4-7 x 10⁷ cpm of [³²P]tRNA^{Phe} judged to be at least 90% pure by fingerprinting analysis.

Replacement of 3' Adenosine with 3'-Deoxy-3'-Aminoadenosine--The substitution of 3'-deoxy-3'-aminoadenosine for A76 was performed essentially as described by Fraser and Rich (19) and is depicted here in schematic form in Figure 1. The 3' adenosine was removed by snake venom phosphodiesterase treatment under conditions where a fraction of C75 was also deleted to ensure complete elimination of A76. The terminus was restored by the step-wise addition of CMP and analogue in separate reactions using *E. coli* tRNA nucleotidyl transferase. The incorporation of CMP was measured in a parallel reaction containing radiolabeled CTP and cold tRNA. 3'-amino-3'-deoxyadenosine was added to tRNA^{Phe} in a 300 µl reaction containing: 50 mM glycine, pH 9.5, 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, 0.75 A₂₆₀ tRNA-CC(OH) containing 7-12 x 10⁶ cpm tRNA^{Phe}, 2.3 mM 3'-deoxy-3'-amino-ATP, and 8.5 units of *E. coli* tRNA nucleotidyl transferase. The reaction was carried out for 75 mins at 37°, stopped by addition of 0.1 vol of 20% potassium acetate, pH 4.5, the protein removed by phenol extraction and the RNA recovered by ethanol precipitation. With this method a minimum of at least 75-80% incorporation was realized. Analogue incorporation was estimated by measuring the amount of [³H]-phenylalanine incorporated into substituted tRNA that is stable to mild alkaline treatment (0.1 M Tris-HCl, pH 9); under these conditions the normal phe-tRNA acyl bond had a half-life of 14 mins.

3'-deoxy-3'-amino-ATP was prepared from the corresponding nucleoside (obtained from the National Cancer Institute) with rabbit liver adenosine kinase as described by Fraser and Rich (19). 3'-deoxy-3'-amino-ATP was separated from unreacted aminoadenosine and ATP by Dowex 1-X2 chromatography using a

Figure 1: Strategy for evaluating the effect of aminoacylation on yeast tRNA^{Phe} using carbodiimide modification. To stabilize the amino acid-tRNA linkage, the 3' terminal adenosine was removed by snake venom phosphodiesterase treatment and replaced with 3'-deoxy-3'-aminoadenosine using tRNA-nucleotidyl transferase. The effect of aminoacylation was analyzed by comparing the T1 RNase fingerprints of carbodiimide modified [³²P]-tRNA with and without amino acid. The RNA samples were dialyzed and modified by carbodiimide in solution containing 10 mM sodium borate, pH 8, 1 or 10 mM Mg²⁺, and ionic strength of 0.1 M.

LiCl gradient. The 3'-deoxy-3'-amino-ATP was then freed of any residual ADP (which co-elutes on Dowex) by conversion to ATP with pyruvate kinase and phosphoenol pyruvate and re-chromatography on the Dowex column. Using this method about 70-75% of the 3'-deoxy-3'-aminoadenosine was converted to 3'-deoxy-3'-amino ATP.

Preparation of Enzymes and Aminoacylation of tRNA--Rabbit liver adenosine kinase was prepared according to the method of Lindberg *et al.* (20), *E. coli* tRNA nucleotidyl transferase according to McGann and Deutscher (21)--except that the cells were disrupted by a different method (22), and crude yeast phenylalanine tRNA synthetase using differential ammonium sulfate precipitations (23). Up to 1 A₂₆₀ of purified tRNA^{Phe} was aminoacylated in a 500 µl reaction mix containing: 100 mM Tris-HCl, pH 7.5, 50 mM MgCl₂, 15 mM ATP, 10 µM phenylalanine, tRNA^{Phe} and up to 2 units of synthetase. Acylation reac-

tions were carried out for 45 min at 37°, stopped by addition of 0.1 vol cold, 20% potassium acetate, pH 4.5, and deproteinized by phenol extraction. For carbodiimide modifications the tRNA was then freed of residual ATP by DEAE column chromatography. Phenol was first removed from the aqueous phase by ether extraction and the RNA adsorbed onto a small (500 µl) DEAE column in a pasteur pipet previously equilibrated in 10 mM sodium acetate, pH 4.5, 10 mM MgCl₂, 0.3 M NaCl. ATP was washed out with the same buffer and the phe-tRNA recovered by elution with 1 ml of the same buffer made 1.0 M in NaCl and precipitation with 3 vols of ethanol.

Reaction with Carbodiimide and Identification of Modification Sites--In a typical modification reaction, 2×10^6 cpm of [³²P]tRNA^{Phe} was mixed with 0.5 - 1.0 A₂₆₀ of unlabeled tRNA^{Phe} and dialyzed extensively against solutions containing 10 mM sodium borate, pH 8, 1 or 10 mM MgCl₂ and NaCl to bring the total ionic strength to 0.1 M. Following dialysis the tRNA was reacted with carbodiimide in a 155 µl reaction mix containing the same buffer, 50 mM carbodiimide (freshly prepared) and MgCl₂ and NaCl to bring the magnesium concentration and ionic strength up to that used in the dialysis. Reactions were carried out for 48 hr at 25°, stopped by addition of 0.1 vol 20% potassium acetate, pH 4.5 and the tRNA recovered by alcohol precipitation. The sites and extents of modification were determined by fingerprinting of T1 RNase digests (24,25) and quantitation of the resulting oligonucleotides. Full details will be provided elsewhere (Fritzinger and Fournier, in preparation). Generally, $0.5 - 2 \times 10^6$ [³²P]tRNA was digested in a reaction mixture containing, in 5 µl, 50 nmoles Tris·HCl, pH 7.4, 5 nmoles EDTA and 30 units RNase T1. Following fingerprinting the individual oligonucleotides were located by autoradiography, excised, and quantitated by Cerenkov counting. Where indicated, oligonucleotides were recovered by elution with 30% triethylammonium bicarbonate (TEC), pH 10, freed of residual TEC by several cycles of evaporation and solubilization and subjected to base compositional analysis by the method of Ohashi *et al.* (26); analyses with pancreatic RNase were carried out as described by Brownlee (27).

RESULTS

Comparison of Normal and Analogue-containing tRNA--Before analogue-containing tRNA could be used to compare the structures of aminoacylated and non-acylated tRNA, it was first necessary to evaluate the effect of substituting

the 3'-deoxy-3'-aminoadenosine for the normally occurring terminal adenosine¹. There were already three lines of evidence indicating that the solution structures of tRNA^{Phe}_{CCA(3'OH)} and tRNA^{Phe}_{CCA(3'NH₂)} are very similar and possibly identical save for the obvious difference. First, it had been demonstrated that tRNA^{Phe}_{CCA(3'NH₂)} is aminoacylated with kinetics almost identical to that of tRNA^{Phe}_{CCA(3'OH)} and that amino acid-containing tRNA^{Phe}_{CCA(3'NH₂)} binds normally to the A-site of the ribosome under standard assay conditions (17). The latter result implies that the ribosome is unable to distinguish between tRNA^{Phe}_{CCA(3'OH)} and tRNA^{Phe}_{CCA(3'NH₂)} even though it can discriminate between tRNA and aminoacyl-tRNA. Second, comparisons of the binding of oligonucleotides complementary to different regions of yeast tRNA^{Phe} did not reveal differences in the binding constants for tRNA^{Phe}_{CCA(3'OH)} and tRNA^{Phe}_{CCA(3'NH₂)} and (9). Finally, the high field proton NMR spectra of tRNA^{Phe}_{CCA(3'OH)} and tRNA^{Phe}_{CCA(3'NH₂)} were compared and found to be identical over the temperature range 30-90° (4). Taken together the results from these studies indicate that incorporation of the analogue has little, possibly no effect on the structure.

In the current study, the structures of tRNA^{Phe}_{CCA(3'OH)} and tRNA^{Phe}_{CCA(3'NH₂)} were compared by carbodiimide modification. The results indicate that the structures are indeed very similar, but in fact, not identical. While the modification patterns for the two forms showed a high degree of qualitative and quantitative similarity two minor differences were observed, one qualitative, the other quantitative. In normal tRNA^{Phe} five nucleotides are readily available for modification, namely: D16 and G20 in the D-loop, U33 and Gm34 in the anticodon loop and U47 in the region of the extra arm. These sites are shown in Figure 2. The extents of modification have been shown to vary with position and solution conditions from 20-90% (28, in preparation, and current study). These same nucleotides are also modified in tRNA^{Phe}_{CCA(3'NH₂)} and to similar extents.

The two differences in modification between tRNA^{Phe}_{CCA(3'OH)} and tRNA^{Phe}_{CCA(3'NH₂)} are highlighted in Table I in the quantitative documentation of T1 RNase products and shown in the final summary figure (Fig. 2). First, incorporation of the adenosine analogue resulted in the appearance of one new modified oligonucleotide. This fragment, identified as G*20AG*AG was caused by new modification of G22 as well as the expected reaction of G20. This new

¹Despite the difference in bonding, all amino acid-containing tRNA will be referred to as aminoacylated for convenience. The nature of the 3'-A will be clearly identified in each case.

Figure 2: Effects of aminoacylation and incorporation of 3'-deoxy-3'-aminoadenosine on the modification of yeast tRNA^{Phe} by carbodiimide.

The modification sites are identified with arrows and, in one case, a bar. Closed arrows designate sites modified in normal, non-acylated tRNA and analog-containing tRNA with and without amino acid. The open arrow identifies a nucleotide modified in analogue-containing, but not normal tRNA. The thin arrow and solid bar denote a single nucleotide and T1 oligonucleotide respectively that are modified only in aminoacylated tRNA. a) Location of sites of modification in the cloverleaf structure of tRNA^{Phe}. Tertiary hydrogen bonds are depicted by lines connecting nucleotides. b) sites of modification as they appear in the tertiary structure.

derivative was found at low yield at about 8%. The basis for the identification is as follows: Base compositional analysis showed the oligonucleotide contained only A and G residues. Only two regions exist in yeast tRNA^{Phe} where modification of a G residue could produce a T1 RNase fragment of this composition, 1) modification of an interior G in the sequence G18GGAGAG in the D-loop and stem and 2) modification of G43 in the sequence G42GAG in the anticodon stem and variable loop. Modification of G43 would produce an oligonucleotide with the sequence G*AG. This product is already present in the fingerprints of normal tRNA^{Phe} owing to modification of G20 in the D-loop (28,29, and in preparation). Accordingly, the appearance of the modified oligonucleotide must be caused by modification of the T1 RNase fragment from the D-loop and stem. Since G20 is already highly modified, the unique oligo-

nucleotide must arise from modification of G19 or G22. The new nucleotide migrates significantly slower than G*AG in both dimensions of a fingerprint consistent with its sequence being G*AG*AG, derived via the modification of both G20 and G22. As shown in the tertiary structure in Fig. 2, G22, though involved in both secondary and tertiary hydrogen bonds, is at the top of the stack of paired bases containing the D and anticodon stems. In this location it is more likely to become available for modification than a residue in the middle of the stack.

The second difference detected between normal and analogue-containing tRNA involved an altered response to magnesium. Our earlier modification analysis of non-acylated tRNA revealed changes in the extent of reaction of the D and anticodon loop nucleotides caused by increasing the magnesium concentration from 1 to 10 mM. This change caused a 25% reduction in the extent of modification of D16 and a 30% increase in the modification of G20 (28 and in preparation). The latter two magnesium-dependent changes were not seen with tRNA^{Phe} CCA(3'NH₂) suggesting that the mobility of the D-loop may be reduced as a consequence of replacing the terminal A with the analogue.

Even with these differences, the results are still consistent with the view that the solution structures of tRNA^{Phe} CCA(3'OH) and tRNA^{Phe} CCA(3'NH₂) have a high degree of similarity. While the modification findings indicate that the structure of the D-loop is changed subtly by incorporation of the analogue, results of the functional and NMR spectral studies identified above—especially the aminoacylation and ribosome binding assays, suggest that this change should not mitigate functionally important changes associated with acylation. For these reasons, it seemed reasonable to suppose that aminoacylation-dependent changes which take place in normal tRNA would likely also occur with analogue-containing tRNA. Conversely, acylation dependent changes observed with tRNA^{Phe} CCA(3'NH₂) would presumably reflect effects experienced by normal tRNA^{Phe}.

Effect of Amino Acid Incorporation on Modification—Aminoacylation caused both qualitative and quantitative changes in the carbodiimide modification pattern. The results are detailed in Table I and highlighted there and in the tRNA structure shown in Fig. 2. The qualitative changes were seen as the appearance of two new, carbodiimide modified oligonucleotides in the T1 RNase fragments indicating that new regions of the tRNA become accessible. The quantitative change was an increase in modification of the site (G22) newly modified in tRNA^{Phe} CCA(3'NH₂) but not in normal, non-acylated tRNA. No effect of magnesium concentration was seen on any of these modifications. With one exception aminoacylation did not change the relative extents of modification of

the five other bases modified in both non-acylated and acylated tRNA. Details of the amino acid-dependent changes follows:

Analysis of the fingerprints of modified tRNA^{Phe}_{CCA(3'NH₂)} and phe-tRNA^{Phe}_{CCA(3'NH₂)} revealed that two new carbodiimide oligonucleotides were seen with aminoacylated tRNA that were not present in the non-acylated species (fingerprints not shown--see Table I). The yields of these oligonucleotides were low and variable, ranging from less than 5% reaction to molar yields of 0.15 to 0.20 indicating extents of modification of 15 to 20%. The latter levels were some 3-4 times that required for detection. The low yields made

TABLE I--MOLAR YIELDS OF RNase T₁ OLIGONUCLEOTIDES

Oligonucleotide	Theoretical Yield	tRNA ^{Phe} _{CCA(3'NH₂)}		phe-tRNA ^{Phe} _{CCA(3'NH₂)}	
		1 mM MgCl ₂	10 mM MgCl ₂	1 mM MgCl ₂	10 mM MgCl ₂
Gp	4	3.30 ± 0.31	3.34 ± 0.23	3.10 ± 0.17	3.10 ± 0.20
Cm ₂ Gp	1	0.71 ± 0.18	0.62 ± 0.15	0.54 ± 0.03	0.54 ± 0.03
CGp	1	1.33 ± 0.22	1.65 ± 0.37	1.34 ± 0.08	1.33 ± 0.18
AGp	3	3.26 ± 0.16	3.15 ± 0.06	3.08 ± 0.08	2.83 ± 0.11
CCAGp	1	1.27 ± 0.01	1.11 ± 0.02	1.01 ± 0.10	0.84 ± 0.27
Cm ₂ CCCAGp				0.25 ± 0.04	0.43 ± 0.02
UGp	1	1.07 ± 0.24	1.02 ± 0.06	1.11 ± 0.07	1.07 ± 0.16
pGp	1	1.03 ± 0.21	1.10 ± 0.11	0.84 ± 0.15	0.92 ± 0.23
DDGp	1	0.75 ± 0.01	0.73 ± 0.07	0.71 ± 0.06	0.67 ± 0.04
CUCAGp	1	1.05 ± 0.05	1.10 ± 0.08	1.06 ± 0.03	1.04 ± 0.03
m ⁷ GUCm ⁵ CUGp	1	0.18 ± 0.02	0.16 ± 0.01	0.22 ± 0.02	0.18 ± 0.01
m ¹ AUCCACAGp	1	0.95 ± 0.04	0.94 ± 0.11	0.96 ± 0.03	0.97 ± 0.03
TvCGp	1	1.19 ± 0.12	1.20 ± 0.04	1.16 ± 0.08	1.18 ± 0.10
AAUUCGp	1	1.16 ± 0.07	1.05 ± 0.03	1.09 ± 0.05	1.10 ± 0.04
AUUUAm ² Gp	1	1.04 ± 0.12	1.08 ± 0.01	1.04 ± 0.08	1.06 ± 0.02
ACmUGmAAAYAm ⁵ CUGp	1	0.31 ± 0.01	0.64 ± 0.07	0.39 ± 0.02	0.73 ± 0.07
(a) ACm(U*) (Gm*)AAAYAm ⁵ CUGp		0.40 ± 0.05	0.22 ± 0.07	0.42 ± 0.04	0.25 ± 0.03
ACmU*Gm*AAAYAm ⁵ CUGp		0.24 ± 0.08	0.11 ± 0.04	0.25 ± 0.02	0.13 ± 0.01
m ⁷ GU*Gm ⁵ CUGp+D*DGp		0.50 ± 0.06	0.63 ± 0.05	0.55 ± 0.17	0.68 ± 0.12
G*AGp		0.50 ± 0.08	0.47 ± 0.04	0.32 ± 0.13	0.28 ± 0.15
(b) (TvCGp)				0.09 ± 0.07	0.12
G*G*AGp				0.15 ± 0.08	0.17 ± 0.04
G*AG*AGp		0.08 ± 0.01	0.07 ± 0.01	0.22 ± 0.11	0.19 ± 0.10

Molar yields of RNase T₁ oligonucleotides derived from digests of carbodiimide modified [³²P] tRNA^{Phe} with and without amino acid. Prior to modification, the 3' terminal adenosine was replaced with 3'-NH₂Ad; tRNA was modified in 1 or 10 mM Mg²⁺ at an ionic strength of 0.1 M. Molar yields were calculated from the expression: molar yield = cpm in oligonucleotide/total cpm in all oligonucleotides x 76 phosphate nucleotides/no. phosphates in oligonucleotide. The yields of modified oligonucleotides were established from the reduced yields of unmodified products and quantitation of new adducts. The results are from two and three independent determinations respectively with non-acylated and amino acid-containing tRNA; the error values correspond to the standard deviation. Incomplete cutting at m⁵G26 was observed in the analyses of phe-tRNA giving rise to the fragment Cm₂CCCAGp; slow cleavage at this site has also been observed by others (29). Some heterogeneity and variation in yields of the expected 3' terminal fragments was observed due presumably to incomplete incorporation of the Ad analog and amino acid or imperfect restoration of the terminus. Sequences in italics correspond to modified fragments or the unmodified parent; asterisks designate the sites of modification. (a) Either U33 or Gm34 is modified. (b) specific site(s) of modification in the TVCG fragment not identified.

rigorous, direct identification of the particular nucleotide adducts difficult. However, results from secondary and tertiary digests and migration position on the fingerprints did provide sufficient information for identification to be made. It is important to note that the effects reported here are reflected primarily in the appearance of new, well resolved modification products in the fingerprints. Because the molar yields of the new products only constitute from 7-22% of the theoretical maximum, a corresponding reduction in yield of unmodified or less modified precursor oligonucleotide is not apparent in each case. This shortfall arises from the fact that some of the losses are close to the pertinent standard deviation.

One of the newly appearing oligonucleotides contained rT and Ψ , indicating that it must arise from modification of the T Ψ CG sequence in the T Ψ C-loop. Owing to low activity the identity of the particular residue modified in this fragment was not determined. However, of the three substrate bases in this fragment the reactive site must be one of the U-derivatives inasmuch as modification of G57 would have protected that site from cleavage by T1 RNase. Because this sequence is not normally available for modification in non-acylated tRNA (28,29, this work and in preparation), this result indicates that aminoacylation causes increased accessibility of the Ψ -loop.

A second carbodiimide modified oligonucleotide unique to phe-tRNA^{Phe} resulted from modification of G19 and G20 in the D-loop and has the sequence G*19G*AG. Base compositional analysis revealed that this modified oligonucleotide contained just A and G residues indicating that it was derived from the D-loop. Since modification of G22 results in the appearance of a different oligonucleotide--seen with non-acylated tRNA^{Phe} CCA(3'NH₂), it seems clear that this new adduct resulted from modification of G19. This assignment is consistent with both the reduced yield of G*20AG seen with modification of phe-tRNA^{Phe} CCA(3'NH₂) and with the position of the oligonucleotide on fingerprints. The yield of this oligonucleotide indicates that the extent of modification of G19 was about 15%. The additional modification of this sequence is correlated with the modification of the T Ψ CG sequence, an indication of weakening of the tertiary interactions between the D- and Ψ -loops.

In addition to the appearance of the new D- and Ψ -loop adducts, aminoacylation also affected the yields of two carbodiimide modified oligonucleotides normally formed in non-acylated tRNA. The yield of the oligonucleotide G*20AG was some 40% lower for phe-tRNA^{Phe} (molar yield $\sim$ 0.3) than for the non-acylated form (molar yield $\sim$ 0.5). This is consistent with an observed 2.5-fold increase in yield of the oligonucleotide G*20AG*AG from 8 to 20% and

the appearance of the oligonucleotide G*19G*AG; both are derived from the same portion of the D-loop (See Fig. 2).

Effect of Magnesium on Modification--The results in Table I also show the effect of magnesium on modification, undertaken to gain insight into the magnesium-mediated change in diffusion which accompanies aminoacylation. Interestingly, no acylation-dependent effects were revealed when the modification patterns of non-acylated and acylated tRNA were compared for 1 and 10 mM Mg^{+2} . It was under these conditions that the diffusion coefficients of acylated tRNA changed by 0 and 18% respectively relative to non-acylated tRNA (albeit at pH 7.2 not the pH 8 condition used here-1).

As seen earlier, changing the magnesium concentration did affect the extent of modification of the anticodon bases U33 and Gm34 (28 and in preparation). In both acylated and non-acylated tRNA^{Phe} modification of this sequence was some 80-100% greater in the lower magnesium condition. That aminoacylation does not effect the extent of modification of these bases in either condition suggests that this region of the tRNA does not change with the change in functional state. Significant magnesium effects ranging from -25% to +50% were also observed with non-acylated tRNA for modification of D16 and G20 in 1 and 10 mM Mg^{+2} , but not here for the analogue-containing isomer-- regardless of the acylation state. Neither did magnesium appear to influence the yields of modified TYCG and G19GAG which were detected only with acylation. This last conclusion must be considered tentative however, owing to the lower precision of the data stemming from the variability in yields of these adducts.

DISCUSSION

The changes in the patterns of modification associated with aminoacylation provide further evidence of a change in solution structure and additional insight into the nature of the change. Two sites are modified in aminoacylated tRNA^{Phe} that are not modifiable in non-acylated tRNA, a residue in the TYCG sequence in the Ψ -loop and G19 in the D-loop. Both modifications indicate that at least partial disruption of the tertiary interactions between the D- and Ψ -loops occurs as a consequence of acylation. In addition, aminoacylation resulted in a 2.5 fold increase in the reactivity of G22 at the end of the D stem. As shown in Fig. 2 all three of these sites are relatively close to each other in the crystal structure, lying near the apex of the "L"- shaped structure. Accordingly, it follows that acylation must cause a change in this region which results in increased exposure of the D- and Ψ -loops. The

relatively low levels of modification of these residues (<20%) makes it unlikely that the structural change is a major metastable rearrangement in which the association of the D- and Ψ -loops is completely disrupted. Were this the case higher extents of reaction would be expected. Furthermore, complete disruption of the tertiary bonds at the junction would presumably also be accompanied by exposure of additional sites of reaction. That new modification is limited both in number of sites and extent argues that the conformational effect is either localized and relatively small or of a dynamic nature.

There are a number of ways in which increased exposure of the junction sequences could occur. First, it is possible that acylation induces subtle localized conformational changes in either or both the D- and Ψ -loops which, consequentially cause a weakening of the tertiary interactions between these two loops. Another possibility is that aminoacylation causes a change in dynamic flexing of domains in the region of the apex, resulting in a decrease in half-time of the tertiary-level interactions between the two loops. Finally, some combination of stable and dynamic conformational changes could provide the basis for the increased exposure. All of these models are consistent with the results observed and the results do not favor selection of one over the others.

Our understanding of the aminoacylation-dependent conformational change is further improved when these findings are considered in context with results obtained by others. Since one of the objectives of this study was to gain insight into the nature of the acylation-dependent diffusion change observed earlier by us the relationship of these two studies will be discussed first and in most detail.

In the light scattering analysis it was determined that aminoacylation is accompanied by an 18% decrease in the translational diffusion coefficient at magnesium concentrations greater than 2 mM; no change was observed at lower magnesium levels (1). The acylation-dependent changes detected by modification, however, showed no magnesium dependence. Taken together the two results appear to be inconsistent. However, it should be remembered that the techniques used in the two studies are sensitive to different aspects of the structure and, of course, different tRNA forms were used in the two investigations.

Chemical modification is sensitive to the intrinsic structure of the tRNA and can detect both global and localized changes in conformation. The laser light scattering method on the other hand, measures translational diffusivity, a hydrodynamic property effected both by the size and shape of the tRNA

and by the shield of counterions and water molecules associated with it. Furthermore, the light scattering method could well be sensitive to dynamic changes whereas the modification method may not. Because these techniques are sensitive to different properties, inconsistencies may actually provide helpful insight into the changes that occur.

The following model is consistent with both sets of results. Specifically, aminoacylation could result in a destabilization of the tRNA near the apex which is reflected as an increase in the modification of the Ψ - and D-loop bases in the current study. But, the molecular events which lead to the altered modification behavior need not cause a change in diffusion behavior. The acylation-dependent structural change, be it metastable or dynamic could, however, effect the magnesium binding properties of the tRNA. Additional binding sites could be created that are largely vacant in 1 mM MgCl_2 , but occupied at concentrations above 2 mM. The binding of extra magnesium, estimated to be between 2 and 4 in the light scattering analysis, could in turn induce additional changes in the structure that do effect the transport properties. Since no further changes in modification occur in 10 mM MgCl_2 , it can be reasoned that the magnesium induced change in diffusivity does not involve long time exposure of new modifiable residues. More likely, the change in diffusion reflects a change in the rate or extent of dynamic flexing or in the distribution of counterions and water associated with the RNA.

This model is consistent with results from NMR and x-ray scattering studies which demonstrated that aminoacylation altered the magnesium binding properties of tRNA (30,31). It is also consistent with results from ribosome binding analyses which suggest the existence of two conformers of aminoacyl-tRNA with altered binding properties (32). In this latter study increasing magnesium concentration favored the formation of a higher affinity form, implying that the magnesium dependent change in diffusivity seen by us may be important in ribosome binding.

One difference between this study and most others on the effect of aminoacylation is the use of tRNA containing the adenosine analogue, 3'-deoxy-3'-aminoadenosine. While the results of earlier work comparing normal and analogue-containing yeast tRNA^{Phe} indicated that the structures are very similar and possibly identical functionally, the modification analysis described here revealed that at least one localized difference exists between the two structures. Although the earlier functional studies demonstrated that the analogue does not interfere with acylation or ribosome binding (17) it is possible that

incorporation of the analogue could influence the conformational change caused by acylation, either damping or amplifying the effect. Of possible special significance is the finding here that the modification of D16 and G20 become insensitive to magnesium on replacement of the 3'-adenosine with the analogue. The changes in modification of these bases in normal tRNA could be due to differential binding of magnesium at or near these sites. This suggestion is consistent with a recent map of magnesium binding sites in yeast tRNA^{Phe} developed by crystallography (33). In that work tRNA^{Phe} was shown to possess six high affinity Mg⁺² sites, two of which are within the D- and Ψ-loop sequences which define the apex region. The loss of the Mg⁺²-induced modification effect with tRNA^{Phe}_{CCA(3'NH₂)} could reflect a change, possibly loss, in magnesium binding in this region. If the magnesium-induced change in diffusivity arises from new or altered binding of Mg⁺² to this very same region, then the absence of a Mg⁺²-effect on modification of phe-tRNA^{Phe}_{CCA(3'NH₂)} could be due to incorporation of the analogue.

The findings of this study demonstrate that aminoacylation results in tertiary level changes in the solution structure of yeast tRNA^{Phe} in the region of the junction of the D- and Ψ-loops. These results are consistent with the majority of the findings described by others. In this regard, little or no acylation-dependent effects have been observed with techniques primarily sensitive to gross conformational changes. This category includes such techniques as circular dichroism (34-38), optical rotatory dispersion (39), tritium exchange (3,40,41), and nuclease digestion (42). The results of the current study are consistent with these results in that the structural changes observed are believed to be small and relatively localized or of a dynamic nature.

Studies using techniques sensitive to small, localized changes have usually been able to detect aminoacylation-dependent changes. Changes in the environment of 4-thiouridine were detected by comparing the CD spectra (from 320-360 nm where s⁴U absorbs) of aminoacylated and non-acylated tRNA (43). Spin labeling studies have also detected changes in this region and in the variable loop (5). Such effects could well be caused by minor changes in structure at the apex, since both s⁴U8 and the variable loop are in a region that could be affected by changes in the D- and Ψ-loops. Results from spin label studies also suggest that aminoacylation may cause changes at the CCA terminus (10), while no change in the structure of the anticodon loop has been seen, either in spin label studies (5) or in Y-base fluorescence analyses (44). The results of the current study are consistent with these results

since no change in the anticodon loop was detected. Inasmuch as the CCA terminus lacks nucleotides reactive to carbodiimide, detection of a change in this region of the tRNA was precluded in the current study.

Others have also observed aminoacylation-dependent changes in the structure of the D- and/or Ψ -loops. Using high resolution NMR Davenloo *et al.* observed differences in the methyl and methylene protons of yeast tRNA^{Phe} CCA(3'NH₂) with acylation (4). These workers suggested that this change was caused by an alteration in the stacking of T54 with adjacent nucleotides resulting in a less stable conformation for this nucleotide. The model in the current study is consistent with such a change.

In a number of ligand binding studies aminoacylation of certain tRNA was accompanied by a dramatic increase in the binding of steroid hormones, presumably to a series of at least three G residues common to the D loop; an increase in the binding of oligonucleotides complementary to the D- and/or Ψ -loops has also been observed (6-8). In all of these studies it was suggested that the increase in ligand binding was caused by a conformational change in which the tertiary interactions between the D- and Ψ -loops were weakened or completely disrupted thereby exposing the loop sequences for ligand association. Complete loss of the apex-specific tertiary bondings is not consistent with the results reported here or with other results alluded to above. However, the ligand binding results could be consistent with either a dynamic change where aminoacylation decreased the half-life of the pertinent tertiary interactions or a model where stable, but localized changes occur in the conformation of these loops. A conformational change could be caused or accompanied by a weakening of the interactions between the loops making the residues in the loops more accessible. Subsequent binding of the ligands could then cause a further change in conformation or a shift in dynamic equilibrium leading to final disruption of the tertiary bonds between the two loops.

A number of other studies also provided evidence that aminoacylation can change tRNA structure although the techniques used do not offer specific information about the structural basis of the change observed. This class includes analysis by sedimentation (2,3), laser Raman spectroscopy (13), laser light scattering (1,11,12), ethidium bromide binding (45), ion exchange chromatography (46), ribosome binding (47), and inhibition of transformylation (48). In all of these studies, the changes reported were small in magnitude and consistent with the scheme developed here.

In several studies no effects were detected on aminoacylation although the techniques used should be sensitive to small structural changes. It is

possible that some studies were conducted under solution conditions that may mitigate acylation-dependent changes. For example, in sodium bisulfate modification studies of Lowdon and Goddard (49) and Negishi *et al.* (50), no detectable change in modification occurred with aminoacylation. However, the reagent used required that modification be performed at low pH (5.0-6.0) and very high ionic strength ($>1.0M$), conditions that may prevent the pertinent structural changes from taking place. In an NMR analysis, no difference was observed in the spectra of tRNA^{Phe} and phe-tRNA^{Phe} (51). However, that analysis was performed at pH 5, again a condition that may not allow an acylation-dependent change to occur. This view is supported by results from a sedimentation study in which an acylation-dependent change was observed for some tRNAs at pH 7 (slower), but no differences were detected at pH 5 (3).

The fact that the normally masked sequences in the apex region become more accessible for modification and for oligonucleotide binding suggests a possible mechanism by which ribosomes are able to discriminate between acylated and non-acylated tRNA. When the apex sequences are paired with each other binding may not be favored. The increase in accessibility of these sequences with acylation might facilitate interaction with specific ribosomal components. This interaction in turn could provide additional stability for specific binding. For several years a popular notion held that anchoring involved interaction of the common TΨCpu sequence in the ψ -loop with a complementary sequence in 5S RNA. However, new results indicate that that interaction is not essential for protein synthesis (52). Of course, that particular interaction may well occur and be important for a ribosome function other than peptide elongation.

A number of proteins are able to distinguish between aminoacylated and non-acylated tRNA. These include the aminoacyl-tRNA synthetases, initiation and elongation factors, ribosomal proteins and tRNA^{Met} transformylase. While some probably recognize and interact with the amino acid, it seems likely that discrimination between the two forms could also depend on a structural change of the type discussed here.

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