Chemical Modification Analysis of Ion-Dependent Changes in the Solution Structure of Yeast Phenylalanine Transfer Ribonucleic Acid†

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ABSTRACT: Chemical modification was used to study major, anomalous changes in the diffusion behavior of yeast phenylalanine tRNA caused by changes in ionic strength and magnesium concentration, all at near-physiological solution conditions. The main chemical probe used was the uracil- and guanine-specific reagent 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, commonly referred to as carbodiimide; some analyses were also performed with the G-specific agent kethoxal (α-keto-β-ethoxybutyraldehyde). Earlier studies in our laboratory revealed the surprising finding that the translational diffusion constant of yeast $tRNA^{Phe}$ is from 6% to 14% higher in solutions of 0.1 M ionic strength containing 1 or 10 mM Mg^{2+} than under conditions of higher ionic strength. Consistent with the earlier conclusion that the diffusion behavior reflects conformational change was the finding here that chemical modification is also altered under these solution conditions. The carbodiimide modification patterns of [^{32}P]$tRNA^{Phe}$ were analyzed under four solution conditions yielding conformers of either low or high diffusivity to gain insight into the structural basis for these changes. The conditions analyzed were 1 or 10 mM Mg^{2+} at μ = 0.1 M or 0.25 M. Comparison of the modification patterns showed that five nucleotides were modified under all conditions, specifically, D16 and G20 in the D loop, U33 and Gm34 in the anticodon loop, and U47 in the variable loop. Major differences were seen in the extents of modification, however, for all but U47, reflecting changes in accessibility or reactivity to the probe. The absence of qualitative differences indicates that the changes in diffusivity do not arise from major, stable rearrangements of the solution structure. Interestingly, little correlation exists between the diffusion and modification behavior. Taken together, the results suggest that the modification differences could occur as a result of localized changes in the D and anticodon loops while the altered diffusion behavior is likely caused primarily by changes in the shape and size of the counterion shield and layer of hydration or by dynamic changes in the structure that cannot be detected by the chemical modification method.

It has been well established that magnesium and monovalent cations are necessary for the formation and maintenance of the three-dimensional structure of tRNA [for reviews, see Cole & Crothers (1978), Schimmel & Redfield (1980), and Teeter et al. (1980)]. It is generally agreed that at ionic strengths greater than 0.1 M and moderate temperatures (25 °C), tRNA occurs in a "native" conformation that likely resembles the crystal structure even in the absence of magnesium; at lower ionic strengths, magnesium is absolutely required for stabilization of the structure (Cole et al., 1972; Reid & Hurd, 1977). Several studies have shown that even at high ionic strength, addition of magnesium results in further stabilization although no obvious conformational change has been identified (Robison & Zimmerman, 1971; Bolton & Kearns, 1977; Labuda et al., 1977).

Most workers agree that there are two classes of magnesium binding sites in tRNA, strong and weak, and that the higher affinity associations can be cooperative or noncooperative [reviewed in Schimmel & Redfield (1980)]. A contrasting view holds that there is only one class of sites, with the apparent differences in affinity being caused by electrostatic effects (LeRoy & Guéron, 1977; Walters et al., 1977). The electrostatic model notwithstanding, results from a variety of solution binding assays indicate that most tRNAs bind between four and six magnesium ions with high affinity, i.e., with binding constants of 10^4–10^5 mol^{-1} (Romer & Hach, 1975; Labuda et al., 1977; Schimmel & Redfield, 1980, and references therein). For some tRNA species, such as *Escherichia coli* $tRNA_f^{Met}$, the number of strong sites may be as small as one (Stein & Crothers, 1976). Six putative magnesium binding sites have been located in yeast $tRNA^{Phe}$ by X-ray crystallography (Holbrook et al., 1977; Jack et al., 1977; Quigley et al., 1978; Teeter et al., 1980). The location of these sites makes it clear that at least some of the bound Mg^{2+} ions serve to neutralize the high concentration of negatively charged phosphates caused by sharp turns in the sugar–phosphate backbone. For this reason, it is likely that at least some of these sites must be occupied by a cation(s) in order for the RNA to assume its native structure in solution. tRNA also binds a larger number of magnesium ions, in the range 20–36, with much lower affinity [$K_a \approx 10^3$ mol^{-1}; reviewed by Schimmel & Redfield (1980)]. These latter sites do not appear to be specific for magnesium but can also be served by monovalent cations such as sodium or by polyamines such as spermine (Teeter et al., 1980).

While salt and magnesium are required for the development and stabilization of the native structure, until recently there has been little evidence of ion-dependent changes in the solution structure at near-physiological conditions. Work in our laboratory has provided evidence that, indeed, magnesium and ionic strength dependent changes do take place with bulk tRNA and for a number of individual accepter species (Olson et al., 1976; Potts et al., 1979; Rhee et al., 1981). In this work, laser light scattering was used to evaluate the diffusion and charge properties of tRNA in different solution conditions with ionic strength and magnesium concentration as variables. The results showed that the translational diffusion coefficient can be markedly influenced by seemingly small changes in magnesium levels. Of special interest was the surprising result that the diffusion coefficient of bulk *E. coli* tRNA was some 11%

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greater in solution that was 1 mM in Mg^{2+} (μ = 0.1 M at pH 7.2, 20 °C) than at conditions of higher magnesium and/or ionic strength. Studies with a number of individual tRNA species from *E. coli* and yeast $tRNA^{Phe}$ showed qualitatively similar results, though the magnitude of the increase in the diffusion coefficient varied (see upper panel Figure 1).

These ion-dependent diffusion changes could occur by any one or a combination of three general mechanisms. First, it is possible that a major metastable rearrangement of the structure with the loss of critical tertiary level bonds is the basis for the altered diffusivity. Another possibility is that one or more relatively small, localized structural changes occur as a consequence of differential ion binding and that these effects in turn cause significant alterations in the size and shape of the counterion shield and water molecules associated with the tRNA. A third possiblity involves changes of a dynamic nature in which the rate or extent of flexing or "breathing" of internal modes provides the basis for the altered transport properties. In the study described here, a chemical modification analysis was undertaken to probe the structures of different conformers to help distinguish among these possibilities.

Chemical modification has proven to be an especially useful method for analyzing the solution structure of tRNA. A number of reagents exist that will react with exposed, unpaired bases, but not with bases involved in secondary or tertiary interactions [reviewed by Brown (1974) and Kim (1978)]. These agents have been used to analyze the structures of a number of different tRNAs. In all cases, the modification patterns are consistent with those expected from the crystal structures, providing evidence that the solution and crystal structures are similar and perhaps identical under the conditions analyzed [for reviews, see Rich & RajBhandary (1976) and Kim (1978)]. Chemical modification probes have also been used successfully to study the thermally induced unfolding of tRNA (Rhodes, 1977), the effect of ribosome and codon binding on tRNA conformation (Herr & Noller, 1978; Wagner & Garrett, 1978, 1979), and, in work to be described elsewhere, a conformational change associated with aminoacylation (Fritzinger & Fournier, 1981, 1982).

Because the diffusion changes of interest occur at low to moderate ionic strengths and neutral pH, it was necessary to identify modification reagents that could be used at these particular solution conditions. Two reagents were selected for use: α-keto-β-ethoxybutyraldehyde (or kethoxal), a guanine-specific probe, and 1-cyclohexyl-3-(2 morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (carbodiimide), which reacts specifically with guanine, uracil, and most modified U residues. The reaction of carbodiimide has been shown to be relatively insensitive to changes in ionic strength and magnesium over the range of solution conditions of interest here (Metz & Brown, 1969). This reagent does show different reactivities with the G[1] and U substrates with the order of reactivity being $\Psi > U > T = G \gg D$ (Brown, 1974; Rhodes, 1975). Yeast $tRNA^{Phe}$ was chosen for the study as it was the only tRNA for which a detailed three-dimensional structure was known at the time the investigation was initiated and because it showed the greatest change in diffusivity (14%) in the laser light scattering experiments (Potts et al., 1979; Rhee et al., 1981). A preliminary account of this work has been presented (Fritzinger & Fournier, 1980).

Materials and Methods

Materials. [^{32}P]Orthophosphate (carrier free) was obtained from New England Nuclear. Preparations of unlabeled yeast $tRNA^{Phe}$ with amino acid acceptor activities in excess of 1150 pmol/A_{260} were either a gift from R. Potts or purchased from Boehringer Mannheim. Bactopeptone and yeast extract were purchased from Difco. Diethylaminoethylcellulose (DEAE-cellulose) was obtained from Whatman and washed with 1 M HCl and 1 M NaOH before use. Benzoylated (BD) DEAE-cellulose was from Boehringer Mannheim and washed extensively with 2 M NaCl and 20% ethanol before use. Acrylamide was purchased from Sigma and Ultra-Pure urea from Schwarz/Mann. Glass-fiber filters (type AP20-25 mm) were obtained from Millipore. Samples of [^{3}H]kethoxal (5.56 mCi/mmol) initially from New England Nuclear were gifts from N. Delihas and M. Litt. Carbodiimide and ribonucleases T_1 and CB were obtained from Calbiochem; pancreatic RNase A was from Worthington. Cellulose acetate strips (3 × 100 cm, CA-Elektrophoresefolien) were from Schleicher & Schuell, and DEAE paper (DE81) was purchased from Whatman. Plastic-backed cellulose thin-layer chromatograms (with fluorescent indicator, 20 × 20 cm) were from Eastman. Electrophoresis tracking dyes (xylene cyanol, acid fuschin, and orange G) were from Fisher. X-ray film (XR-1) was obtained from Eastman Kodak Co. All other chemicals were reagent grade and obtained from standard sources.

Preparation of tRNA. *Saccharomyces cerevisiae* strain D273-10B (α,ρ^+) was obtained from T. L. Mason. Cells were grown in the yeast low phosphate (YLP) medium of Rubin (1974) from which inorganic phosphate had first been precipitated as the magnesium salt by the addition of $MgSO_4$ and concentrated NH_4OH. The precipitate was removed by filtration and the pH of the medium adjusted to 5.8 with HCl. ^{32}P labeling of the cells and isolation of low molecular weight RNA were done essentially according to the method of Rubin (1974). Typically, a 250-mL culture was labeled with 50 mCi of carrier-free [^{32}P]orthophosphate. By use of this method, approximately 5 mCi of ^{32}P was incorporated into the low molecular weight RNA.

[^{32}P]$tRNA^{Phe}$ was purified by a new method that is a combination of BD cellulose chromatography and the two-dimensional gel electrophoresis method of Garel et al. (1977). Following extraction, DEAE-cellulose chromatography, and ethanol precipitation, the low molecular weight RNA was recovered by centrifugation, dried in vacuo, resuspended in water, and applied to a small (0.6 × 7.8 cm) BD cellulose column previously equilibrated in 10 mM sodium acetate, 10 mM $MgCl_2$, and 0.3 M NaCl. After being washed briefly with the same solution, the bulk of the RNA was eluted with the same buffer made 1.0 M in NaCl. The $tRNA^{Phe}$ was then eluted with the acetate–magnesium buffer that was 1.0 M NaCl and 20% ethanol. The fractions (1 mL) containing most of the radioactivity were pooled, and the tRNA was recovered by alcohol precipitation. The RNA was resuspended in 300 μL of H_2O, dried in vacuo, and then resuspended in 15 μL of a 50% urea solution and incubated 5 min at 65 °C to dissociate any aggregates. Ten microliters of a mixture of 40% sucrose and 2% xylene cyanol was added and the sample applied to two 3-cm tracks of a 45 × 14 × 0.15 cm 9.6% polyacrylamide gel [9.1% acrylamide, 0.5% bis(acrylamide)] containing 7 M urea.

Electrophoresis was carried out for 48 h at 450 V (constant voltage) at 4 °C. The region of the gel containing the tRNAs

[1] Abbreviations: U, uracil; G, guanine; C, cytosine; A, adenine; D, dihydrouracil; Ψ, pseudouracil; BD cellulose, benzoylated diethylaminoethylcellulose; μ, ionic strength; SSC (standard saline citrate), 0.015 M sodium citrate and 0.15 M NaCl; TEC, triethylammonium bicarbonate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

was located by autoradiography, excised, and placed at the top of a 14 × 21 × 0.15 cm 20% polyacrylamide gel (19:1) containing 4 M urea. The gel slice (one per second dimension) was then sealed in place with 9.6% gel. The RNAs were further fractionated by electrophoresis for 96 h at 5 mA (constant current) at 4 °C and located again by autoradiography. The region of the gel containing $tRNA^{Phe}$, identified initially by coelectrophoresis with purified unlabeled $tRNA^{Phe}$ and methylene blue staining, was excised, passed through a 10-mL syringe, and extracted with 2 × SSC (1 × SSC = 0.15 M NaCl, 0.15 M sodium citrate, pH 7.0). The $tRNA^{Phe}$ was freed of residual acrylamide by absorption to and desorption from a small (1.5 mL) DEAE column, dialyzed extensively against H_2O, taken to dryness, and resuspended in 200 μL of H_2O. This procedure generally yielded (4–7) × 10^7 cpm of [^{32}P]$tRNA^{Phe}$ that was at least 90% pure based on fingerprinting analysis.

Chemical Modification Conditions. Unlabeled $tRNA^{Phe}$ was prepared for kethoxal modification by first dialyzing against 10 mM Tris–borate buffer (pH 7.2) containing 1 or 10 mM $MgCl_2$ and NaCl to yield a final ionic strength of 0.1 or 0.25 M. Then a portion of the $tRNA^{Phe}$ solution containing 5 μg of RNA was reacted with [^{3}H]kethoxal at 20 °C in a 50-μL reaction that was 10 mM Tris–borate (pH 7.2) and 18 mM [^{3}H]kethoxal and contained $MgCl_2$ and NaCl in the same concentrations as the dialysis buffer. Seven microliter samples were withdrawn from the reaction mix at 0, 0.5, 1, 2, 3, and 4 h and added to 2 mL of cold 5% trichloroacetic acid. After 30 min on ice, RNA precipitates were recovered by filtration through glass-fiber filters, and the filters were dried and counted by liquid scintillation spectrometry.

For a typical carbodiimide reaction, 2 × 10^6 cpm of [^{32}P]$tRNA^{Phe}$ was mixed with 0.5–1.0 A_{260} unit of unlabeled $tRNA^{Phe}$ and dialyzed extensively against one of four solutions. These solutions contained 10 mM sodium borate (pH 8), 1 or 10 mM $MgCl_2$, and sufficient NaCl to make the total ionic strength 0.1 or 0.25 M. Following dialysis the [^{32}P]$tRNA^{Phe}$ was reacted with carbodiimide in a 156-μL reaction mix containing the same borate buffer, 50 mM carbodiimide (freshly prepared), and $MgCl_2$ and NaCl to mimic the solutions against which the RNA had been dialyzed. Except where indicated reactions were carried out for 48 h at 25 °C and stopped by the addition of 0.1 volume of 20% potassium acetate, pH 4.5, and the tRNA was recovered by ethanol precipitation.

Identification of Modification Sites. The sites of carbodiimide modification were determined by fingerprint analysis according to the method of Barrell (1971) as modified by Woese et al. (1976). Per analysis (0.5–2) × 10^6 cpm (less than 1 A_{260}) of tRNA was digested with T_1 RNase for 30 min at 37 °C in a reaction mixture containing, in 5 μL, 50 nmol of Tris-HCl (pH 7.4), 5 nmol of EDTA and 30 units of RNase T_1. After fingerprinting, the individual oligonucleotides were located by autoradiography, excised, and quantitated by Cerenkov counting in water. The radioactive oligonucleotides were recovered by placing the DEAE paper in siliconized Pasteur pipets and eluting with small quantities of 30% triethylammonium bicarbonate (TEC), pH 10. Eluants were collected on parafilm, and residual TEC was removed by several cycles of evaporation and solubilization in 25 μL of distilled water. Where indicated, base compositional analyses were performed according to the two-dimensional thin-layer chromatographic method of Ohashi et al. (1974); in most cases, only the second dimension of the system was used. Pancreatic ribonuclease digestions and separation of the resulting oligonucleotides was performed by high-voltage electrophoresis according to Brownlee (1972).

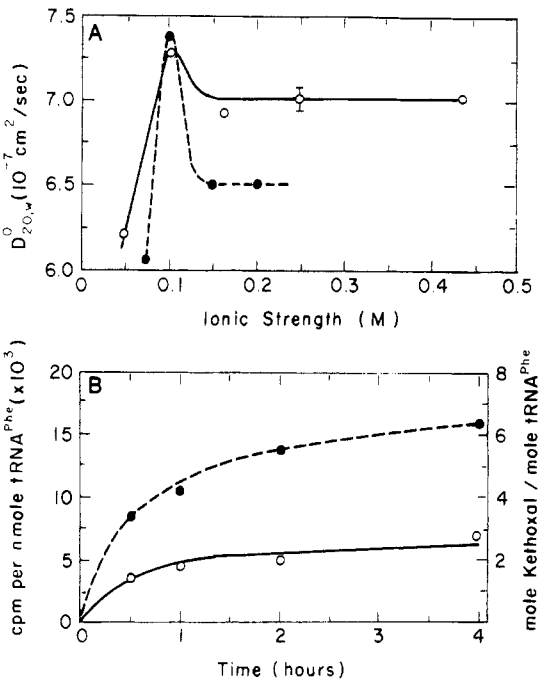


FIGURE 1: Ion-dependent effects on the diffusion and chemical modification behavior of yeast $tRNA^{Phe}$. (A) Effect of ionic strength and magnesium concentration on the translational diffusion coefficient ($D^{\circ}_{20,w}$) at pH 7.2 and 20 °C. The tRNA was dialyzed against buffer in 10 mM $MgCl_2$ and NaCl to achieve the ionic strengths indicated. The diffusion coefficients, $D_{20,w}$, were determined by laser light scattering for four to six different concentrations of RNA at each solution condition. The values shown ($D^{\circ}_{20,w}$) are from extrapolations to infinite dilution. Details are provided under Materials and Methods and the earlier publications cited therein: (●) data obtained for 1 mM $MgCl_2$; (○) data for the 10 mM $MgCl_2$ condition. The error bar represents ±1 standard deviation about the point. (B) Time course of α-keto-β-ethoxybutyraldehyde (kethoxal) modification of $tRNA^{Phe}$ in 1 or 10 mM $MgCl_2$ at 0.1 M ionic strength, pH 7.2, 20 °C. 1 A_{260} unit of $tRNA^{Phe}$ dialyzed against the appropriate solutions was reacted with [^{3}H]kethoxal in a 50-μL reaction mixture containing buffer, 1 or 10 mM $MgCl_2$, 18 mM kethoxal, and NaCl to attain a final ionic strength of 0.1 M. At the times indicated, 7-μL portions were withdrawn and precipitated and the extent of reaction was determined by liquid scintillation spectrometry. (●) 1 mM Mg^{2+}; (○) 10 mM Mg^{2+}.

Results

Kethoxal Modification of $tRNA^{Phe}$. As a first step in utlizing the chemical probes to characterize the changes in diffusivity observed in the light-scattering experiments, yeast $tRNA^{Phe}$ was reacted with [^{3}H]kethoxal under conditions that produce different diffusion behavior. The incorporation reactions were followed by sampling and measuring acid-insoluble radioactivity with time. The ionic conditions evaluated were 1 and 10 mM Mg^{2+} at ionic strengths of 0.1 and 0.25 M. As shown in panel A of Figure 1 the tRNA is in a slower diffusing form in the high salt–low magnesium condition, implying that it is in an extended conformation; under the other conditions tested, the tRNA has a significantly higher diffusion coefficient.

Two patterns of modification were observed for the four conditions evaluated. The results, illustrated for two conditions in panel B of Figure 1, showed that, on average, 2 mol of kethoxal were incorporated per mol of tRNA for all but the low salt–low magnesium condition. In this condition between 5 and 6 mol of kethoxal were incorporated per mol of tRNA. For all but the last condition the modification results are in good agreement with that of Litt (1969), who determined that

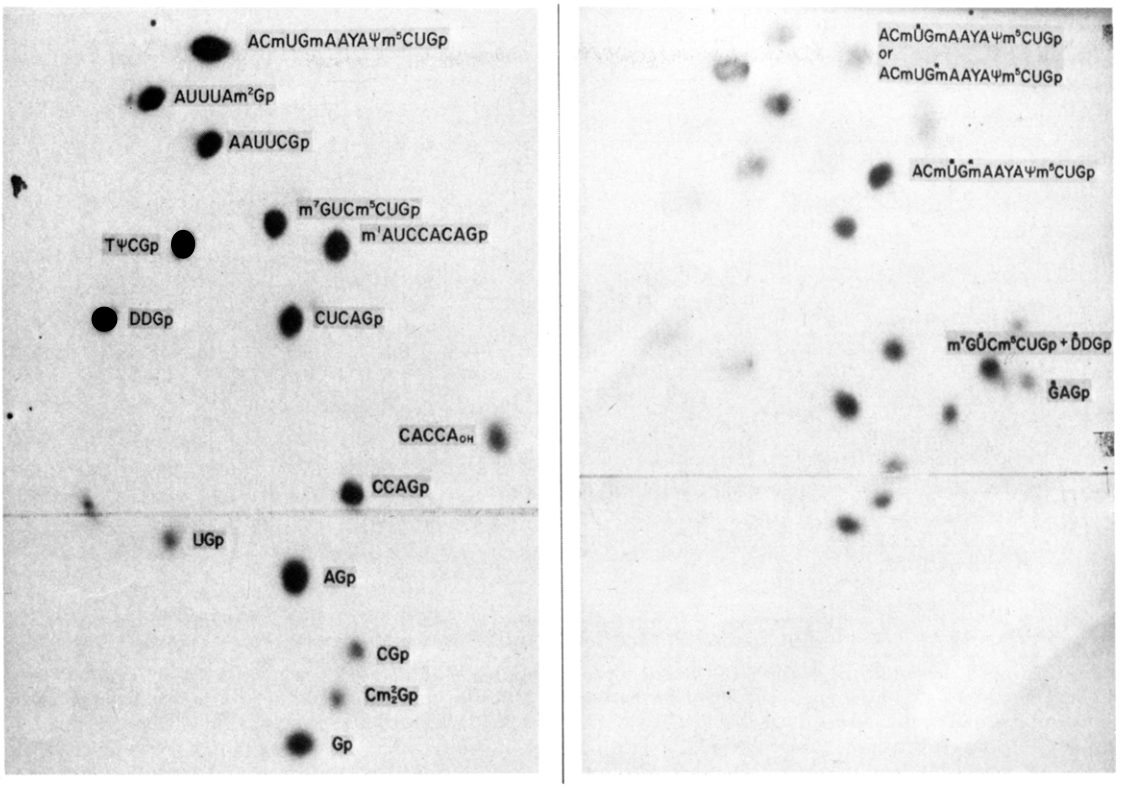


FIGURE 2: Autoradiographs of RNase T_1 fingerprints of unmodified and carbodiimide-modified yeast [^{32}P]tRNA^{Phe}. 10^6 cpm [^{32}P]tRNA corresponding to 50 μg was digested with 30 units of RNase T_1, and the resulting oligonucleotides were separated by the two-dimensional electrophoretic method of Sanger et al. (1965) as modified by Woese et al. (1976). Modification with carbodiimide was carried out in 1 mM Mg^{2+} at μ = 0.1 M. Fingerprints of tRNA modified under the other solution conditions evaluated were similar, though the relative intensities of the modified oligonucleotides vary. The identities of the component oligonucleotides are given: (left) unmodified control tRNA; (right) carbodiimide-modified tRNA.

only G20 and Gm34 reacted with kethoxal under a similar condition (180 mM sodium cacodylate, pH 7, 5 mM Mg^{2+}). The finding that additional G residues are accessible to the modification probe in the low salt–low Mg^{2+} condition provides additional evidence for the occurrence of an ion-dependent change in the solution structure. The dissimilarity of the modification patterns for the two conditions causing the highest (and similar) diffusion values (1 and 10 mM Mg^{2+} at μ = 0.1 M) suggests that different conformers may obtain under these two conditions.

Carbodiimide Modification Analysis. Having determined that the chemical modification pattern can also be influenced by solution conditions, an effort was made to obtain detailed information about the sites of modification with a view to gaining insight into the bases for both the diffusion and modification behaviors. For simplification of the identification of the modifiable nucleotides, in vivo labeled [^{32}P]tRNA^{Phe} was used in subsequent modification reactions. In addition carbodiimide was chosen as the modification probe because of its broader specificity. Since the optimal pH for carbodiimide modification is somewhat higher (pH 8) than that chosen for the original diffusion measurements (pH 7.2), it was necessary to evaluate the diffusion behavior of the tRNA under the new solution condition. The results of this analysis, shown in summary form in Figure 3 (upper left panel), demonstrated that tRNA^{Phe} behaves qualitatively the same at pH 8 and 7.2, though the actual $D^{\circ}_{20,w}$ values do differ from those seen at the lower pH (Figure 1). A larger change in diffusivity was observed at the higher pH condition as the ionic strength was increased from 0.1 to 0.25 M in 1 mM $MgCl_2$; almost no change occurred in the 10 mM Mg^{2+} condition. Because of the similarity in diffusion behaviors at pH 7.2 and 8, it was possible to use the carbodiimide probe at optimum reaction conditions in these experiments.

Carbodiimide modification was performed at the same four ionic conditions identified earlier, namely, 1 mM Mg^{2+} at μ = 0.1 or 0.25 M and 10 mM Mg^{2+} at μ = 0.1 or 0.25 M. The low Mg^{2+}–high salt condition yields a slower diffusing form while faster diffusing but not necessarily identical conformers obtain under the other conditions. It was reasoned that a comparison of the modification patterns for the low and high Mg^{2+} conditions might also reveal effects of differential magnesium binding, especially at the higher ionic strength.

Figure 2 shows typical T_1 RNase fingerprints of unmodified and carbodiimide modified yeast [^{32}P]tRNA^{Phe}. The modification in this particular case was perfomed under the low salt–low magnesium condition. However, tRNA^{Phe} modified under the three other conditions gave qualitatively similar patterns. When the fingerprints are compared, it can be seen that carbodiimide modification caused a visible loss in intensity of three fragments and the appearance of five new oligonucleotides. The oligonucleotides obviously in reduced yield were DDGp in the D loop, ACmUGmAAYAΨm^{5}CUGp in the anticodon loop, and m^{7}GUCm^{5}CUGp in the variable loop and stem of the Ψ loop. Analysis of the oligonucleotides revealed that carbodiimide modified five nucleotides: D16 and G20 in the D loop, U33 and Gm34 in the anticodon loop, and U47 in the variable loop. This finding is in good agreement with the results of Rhodes (1975), who also used carbodiimide as a probe of yeast tRNA^{Phe} and observed modification of the same residues for a single low salt–high Mg^{2+} condition (10 mM Mg^{2+}; μ = 0.035 M). No modification of G18 was detected in the current study whereas slight reaction was observed in the earlier analysis; the difference could be due to the use of different temperatures, 37 °C in the former analysis and 25 °C in the current study. The positions of the

Table I: Molar Yields of RNase T_1 Oligonucleotides[a]

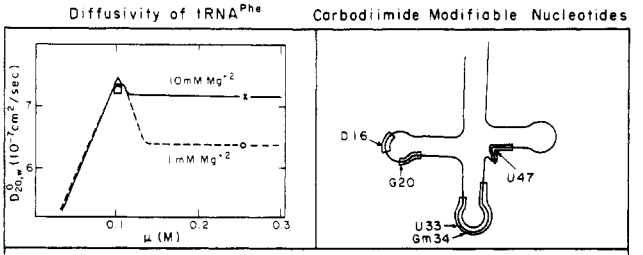
oligonucleotide	modification solution condition: unmodified control	1 mM $MgCl_2$, μ = 0.1 M	1 mM $MgCl_2$, μ = 0.25 M	10 mM $MgCl_2$, μ = 0.1 M	10 mM $MgCl_2$, μ = 0.25 M
Gp	4.09 ± 0.70	3.75 ± 0.70	3.40 ± 0.69	3.28 ± 0.71	3.85 ± 0.77
Cm_2^2Gp	0.90 ± 0.11	1.34 ± 0.42	1.19 ± 0.25	1.15 ± 0.04	1.18 ± 0.04
CGp	1.05 ± 0.11	1.14 ± 0.15	1.08 ± 0.24	1.20 ± 0.16	1.26 ± 0.21
AGp	3.29 ± 0.35	3.42 ± 0.54	3.04 ± 0.33	2.88 ± 0.09	2.99 ± 0.34
CCAGp	0.90 ± 0.11	1.20 ± 0.06	1.05 ± 0.17	0.99 ± 0.05	1.02 ± 0.04
UGp	1.03 ± 0.10	1.23 ± 0.12	1.10 ± 0.23	1.21 ± 0.02	1.10 ± 0.11
pGp	0.95 ± 0.09	1.11 ± 0.14	1.06 ± 0.07	1.04 ± 0.12	1.05 ± 0.08
DDGp	1.04 ± 0.06	0.59 ± 0.11	0.83 ± 0.02	0.68 ± 0.08	0.80 ± 0.01
$CACCA_{OH}$	0.92 ± 0.13	0.72 ± 0.36	0.83 ± 0.09	0.76	0.93
CUCAGp	1.01 ± 0.05	0.91 ± 0.10	1.00 ± 0.08	1.00 ± 0.04	0.90 ± 0.00
m^7GUCm^5CUGp	1.03 ± 0.05	0.27 ± 0.13	0.21 ± 0.11	0.16 ± 0.08	0.21 ± 0.07
$m^1AUCCACAGp$	0.88 ± 0.13	0.82 ± 0.12	0.89 ± 0.04	1.00 ± 0.14	0.92 ± 0.16
TΨCGp	1.18 ± 0.09	1.17 ± 0.03	1.17 ± 0.05	1.05 ± 0.26	1.20 ± 0.13
AAUUCGp	1.06 ± 0.07	1.02 ± 0.14	1.06 ± 0.08	1.11 ± 0.11	1.01 ± 0.07
$AUUUAm^2Gp$	1.08 ± 0.10	0.94 ± 0.18	0.94 ± 0.05	1.01 ± 0.03	0.94 ± 0.04
ACmUGmAAYAΨm⁵CUGp	0.95 ± 0.09	0.16 ± 0.07	0.38 ± 0.15	0.40 ± 0.03	0.47 ± 0.06
ACm(U)(Gm*)AAYAΨm⁵CUGp*[b]		0.30 ± 0.06	0.30 ± 0.03	0.18 ± 0.03	0.20 ± 0.03
*ACmU*Gm*AAYAΨm⁵CUGp*		0.26 ± 0.05	0.16 ± 0.04	0.09 ± 0.05	0.09 ± 0.04
a[c]		0.49 ± 0.21	0.38 ± 0.21	0.24 ± 0.08	0.13 ± 0.06
$m^7GU*Cm^5CUGp+D*DG$		0.71 ± 0.21	0.71 ± 0.25	0.83 ± 0.09	0.75 ± 0.10
*G*AGp*		0.62 ± 0.11	0.56 ± 0.11	0.87 ± 0.25	0.84 ± 0.53

[a] Molar yields of RNase T_1 oligonucleotides derived from digests of unmodified [^{32}P]$tRNA^{Phe}$ and [^{32}P]$tRNA^{Phe}$ modified with carbodiimide under four conditions of ionic strength and Mg^{2+} concentration. Molar yields were calculated by using the formula molar yield = (cpm in oligonucleotide/total cpm in all oligonucleotides)(76 phosphate nucleotides/number of phosphates in oligonucleotide). The yields of modified oligonucleotides were established from the reduced yields of unmodified product and quantitation of new adducts. The results are from three to seven independent determinations involving multiple preparations of RNA; the error values correspond to the standard deviation. Sequences in italics correspond to modified fragment or its unmodified parent; asterisks designate the sites of modification. [b] Either U33 or Gm34 is modified. [c] Product is derivative of 3'-terminal oligonucleotide, $CACCA_{OH}$.

carbodiimide-modified residues in the tRNA are depicted in Figure 3.

Since modification under all solution conditions gave qualitatively similar patterns, it seems clear that the changes in diffusivity observed in light scattering do not occur as a consequence of a major, metastable conformational change. However, examination of the quantitative results revealed a broad range of reactivities for four of the five modifiable nucleotides reflecting the occurrence of both position and solution effects. The quantitative results for all nucleotides are given in Table I and highlighted for the reactive sites both there and in Figure 3. The levels of reaction ranged overall from 17% to 87%, with maximum differences in the extents of modification for a given nucleotide of 15–140% for the four solution conditions evaluated [(max − min)/min × 100)]. The four nucleotides showing the greatest variance were D16, G20, U33, and Gm34; little or no differences were seen in the modification of U47. The data presented were derived from between three and seven experiments and, in most cases, are remarkably consistent, with standard deviations generally less than 10%. Following is an account of the responses of the four variably modified residues.

Dihydrouridine Loop. D16 was modified to the greatest extent (40%) in 1 mM $MgCl_2$, μ = 0.1 M, conditions that cause the formation of a fast diffusing form (Figure 3). A second solution condition producing a fast diffusing conformer, 10 mM $MgCl_2$, μ = 0.1 M, resulted in somewhat lower modification, about 30%. The least modification of D16 (~20%) was seen in high salt in either 1 or 10 mM $MgCl_2$, conditions which yield slow and fast diffusing forms, respectively. These results imply that the modification of D16 is mostly dependent on ionic strength, though at μ = 0.1 M increasing the Mg^{2+} concentration from 1 to 10 mM resulted in a 25% reduction in the modification of D16. Inasmuch as the crystal structure shows D16 to be quite exposed, the somewhat lower extents of modification of this base when compared with the others is probably caused by its inherently lower reactivity (Rhodes, 1975), rather than its availability to solution.



	Δ 1 mM $MgCl_2$ μ = 0.1 M	o 1 mM $MgCl_2$ μ = 0.25 M	□ 10 mM $MgCl_2$ μ = 0.1 M	x 10 mM $MgCl_2$ μ = 0.25 M
	Molar Yield of Modified Nucleotide			
D16	0.41	0.17	0.32	0.20
G20	0.62	0.56	0.87	0.84
U33 and/or Gm34	0.84	0.62	0.60	0.53
U47	0.73	0.79	0.84	0.79

FIGURE 3: Carbodiimide modification patterns for yeast $tRNA^{Phe}$ in different conditions of magnesium and ionic strength. The upper left panel shows the effects of ionic strength and Mg^{2+} concentration on the diffusion behavior of $tRNA^{Phe}$ under the conditions used for carbodiimide modification. Samples were treated and analyzed as described in Figure 1 and under Materials and Methods. These measurements were performed at pH 8 and 25 °C whereas earlier diffusion studies were usually at pH 7.2 and 20 °C. Solid line designates data for 10 mM Mg^{2+}; broken line corresponds to results for 1 mM Mg^{2+}. The symbols identify the four solution conditions used in the carbodiimide modification analysis. The upper right panel shows the position of the RNase T_1 oligonucleotides modified by carbodiimide and the specific nucleotides attacked. The bottom panel shows the molar yields of carbodiimide-modified nucleotide obtained for each of the four solution conditions analyzed. The modification results are taken from Table I.

While the extent of modification of D16 is primarily salt dependent, the variance in modification of G20 is entirely

magnesium dependent. Under both low Mg^{2+} conditions, G20 is modified to an extent of about 60%, while the modification is nearly 50% higher at 85% in 10 mM $MgCl_2$ at either ionic strength evaluated.

Anticodon Loop. The nucleotides in the anticodon loop available for modification, U33 and Gm34, show behavior similar to though not identical with that of D16. The greatest extent of modification (~85%) occurs in 1 mM $MgCl_2$, μ = 0.1 M, a condition where $tRNA^{Phe}$ assumes a rapidly diffusing form. The lowest extent of modification (~55%) was found when modification occurred in 10 mM $MgCl_2$, μ = 0.25 M. Intermediate levels of modification (~60%) were found for the other two conditions tested (10 mM $MgCl_2$, μ = 0.1 M, and 1 mM $MgCl_2$, μ = 0.25 M).

All of the modification results imply that the reactivities of the different nucleotides are affected differentially by changes in the solution conditions. The differences in modification of D16 appear to be primarily salt dependent while the variability in G20 modification is solely dependent on Mg^{2+}. The modification of the anticodon nucleotides is influenced by both salt and magnesium.

Kinetics of Carbodiimide Modification. The data presented in Table I reveal substantial differences in the availability of four of the five carbodiimide modifiable nucleotides, depending on the solution condition. A kinetic analysis of modification was performed to gain additional insight. Because of the difficulty of this method of analysis, the rate study was limited to two time points, 24 and 48 h, and two solution conditions, 1 mM $MgCl_2$, μ = 0.1 M, where the diffusivity is high, and 1 mM $MgCl_2$, μ = 0.25 M, where the D° value is 17% lower. The results (not presented) showed the modification of the U33Gm34 sequence to be largely completed by 24 h and progressing slowly thereafter. While the solution effects on D16 were similar to those for U33/Gm34, both the rate and extent of modification were substantially lower. For both conditions evaluated, D16 modification increased some 2-fold between 24 and 48 h, yielding final extents of 38% and 27%, respectively, for the low and high ionic strength conditions. As noted earlier, the response of U47 differed from that of the others in that solution conditions had little effect on the rate of modification; reaction was largely over by 24 h, with increases of only 10–30% over the second 24-h period. Although limited to only two time points, the results of the rate study do corroborate the earlier findings of differential effects related to both position and solution conditions.

Discussion

Comparison of Kethoxal and Carbodiimide Results. Qualitatively, the kethoxal modification results are in agreement with those obtained by using carbodiimide in that the highest level of modification in both cases occurs in the low salt–low magnesium condition. However, the kethoxal modification data suggest the occurrence of a considerable change in the solution structure under these conditions, in which, based on molar yields of adduct, two to three new G residues are exposed to the environment; the more definitive carbodiimide results suggest that no such change occurs. This apparent inconsistency can be explained in several ways. First, carbodiimide is a much larger, bulkier reagent, being about 3 times the size of kethoxal; in addition, the rate of modification by carbodiimide is about one-tenth that of kethoxal. It is possible therefore that kethoxal could react with particular G residues whose reaction with carbodiimide is sterically hindered, making kethoxal sensitive to changes that cannot be detected with carbodiimide.

Another possible basis for the apparent difference between the modification results is the occurrence of modification-dependent conformational change, where modification itself causes a structural change in the tRNA, resulting in new nucleotides becoming available for subsequent modification. This phenomenon can occur with both carbodiimide and kethoxal as well as other agents (Brown, 1974; Litt, 1969). In the current study this has posed only minor difficulty with carbodiimide where, on very long reaction times (in excess of 72 h), some apparently random modification of $[^{32}P]tRNA^{Phe}$ has been seen (unpublished results). Modification-dependent modification has proven to be more of a problem with kethoxal where at high kethoxal levels (especially) and probably with prolonged reaction a high proportion of G residues eventually become modified (unpublished results).

Yet another possible explanation is a dynamic change in structure in which the tRNA exhibits increased flexing in the low salt–low magnesium condition. In breathing, the kinetics of secondary and tertiary pairings may be changed so that residues previously unavailable for modification become modifiable. Inasmuch as the rate of reaction by kethoxal is more than an order of magnitude greater than that of carbodiimide, dynamic changes may be more readily detected with the kethoxal probe.

That carbodiimide can, in fact, detect at least some localized changes in tRNA is evident from a thermal melting analysis of yeast $tRNA^{Phe}$ (Rhodes, 1977) and a study of the effect of aminoacylation (Fritzinger & Fournier, 1981, 1982). In the denaturation study it was possible to detect early, localized melting originating with the G4·U69 base pair in the interior of the acceptor stem. In the second case carbodiimide was used successfully to detect a conformational change associated with aminoacylation of yeast $tRNA^{Phe}$. In that work both qualitative and quantitative effects were observed. The incorporation of amino acid resulted in new but partial modification of the TΨCG sequence, G19 and G22, indicating that aminoacylation causes increased exposure of residues at the apex of the L-shaped molecule. Both studies serve to demonstrate the ability of this probe to sense at least certain tertiary level changes.

The carbodiimide modification results make it clear that while ion-dependent changes can be identified the alterations which cause the dramatic changes in diffusivity do not cause new regions of the molecule to become stably available for modification. Accordingly, it seems clear that the changes in diffusivity do not occur as a consequence of major, stable rearrangements in the tRNA in which tertiary and perhaps secondary hydrogen bonds are disrupted. Such changes would almost surely lead to the exposure of new G or U residues, a condition which should be readily detected by modification.

Although the results with carbodiimide effectively rule out any major rearrangement yielding a different, metastable form, it is clear that large changes in the extent of modification of some nucleotides do occur. These differences are believed to be due to changes in the solution structure rather than changes in the reactivity of carbodiimide. First, no significant change is seen in the reactivity of U47 under conditions that cause significant differences in the reactivities of the other nucleotides modified. Second, in studies performed on mononucleotides and oligonucleotides, Metz & Brown (1969) observed that carbodiimide modification was quite insensitive to changes in magnesium concentration from 0 to 10 mM and ionic strength over the range 8.3–100 mM. The differences in reactivity observed here then must reflect changes in the accessibility and/or electronegative potential of the nucleotides caused by

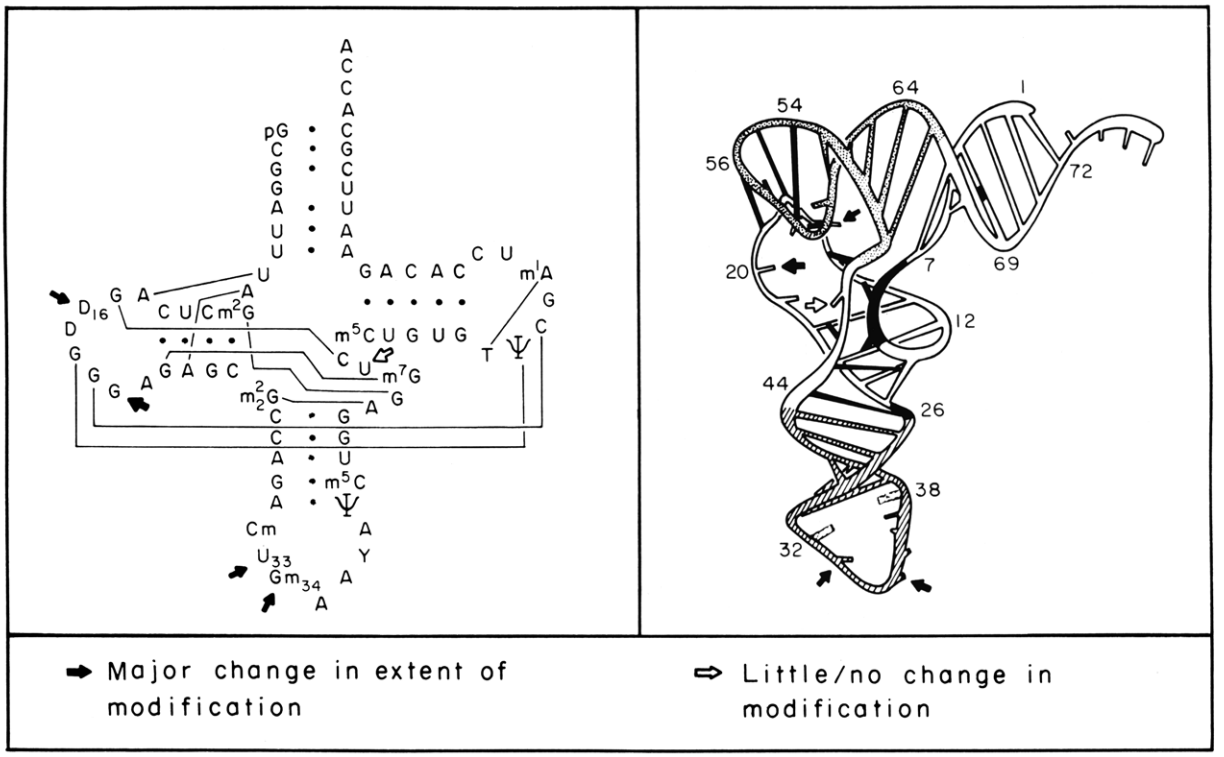


FIGURE 4: Carbodiimide modification behavior for yeast $tRNA^{Phe}$ under varying conditions of Mg^{2+} and ionic strength. The modification sites are identified with arrows. Closed arrows designate sites exhibiting major change in extent of modification over the range of ionic conditions tested. The open arrow denotes a site where the level of reactivity is relatively unaffected by change in the ionic conditions.

alterations in the tRNA structure.

It may be helpful to note that two other chemical probes which will be useful in studies of RNA solution structure were identified while this work was in progress. Peattie & Gilbert (1980) demonstrated that the reagents dimethyl sulfate and diethyl pyrocarbonate can also be used to detect changes in the solution structure of yeast $tRNA^{Phe}$ under near-physiological conditions. With these agents it was possible to detect changes in both secondary and tertiary structure associated with the removal of magnesium and complete denaturation by heat.

Comparison of Modification and Diffusivity Results. Since the modification experiments were initially undertaken to gain insight into the bases for the observed changes in diffusivity, it is useful to compare the results from each method of analysis for the same solution conditions to determine if correlations exist. This is done for the carbodiimide results in Table II. Examination of these results makes it clear that, in fact, there is little or no correlation. In three of the four solution conditions examined, the tRNA occurs in a rapidly diffusing form with similar diffusion constants. However, the modification of D16, G20, U33, and Gm34 varied greatly over these conditions (D16, 100%; G20, 40%; U33 and Gm34, 58%). Similarly, a comparison of the modification results for the condition favoring low diffusivity with the results obtained for the other conditions also fails to reveal consistencies. The lack of correlation between the modification and diffusion data must be due either to the existence of different tRNA conformers with similar diffusivities or to the possibility that stable changes in the intrinsic molecular structure per se are not the major effect contributing to the altered diffusion behavior.

Solution Effects on Modification. It is clear that the modification behavior of U33 and Gm34 is related to both the ionic strength and magnesium concentration of the solution (Tables I and II). In the crystal structure of $tRNA^{Phe}$, shown in Figure 4 with the sites of modification identified, Gm34 is highly exposed to the environment and should be readily available for modification under all conditions examined. U33 is less available since it is folded into the interior of the anticodon loop and is hydrogen bonded to phosphate 36 (Quigley & Rich, 1976). Despite this orientation, however, it is reasonable to expect that it too should be modified under all conditions studied. The results reported here indicate that the modification of U33 and Gm34 is affected by solution conditions. The differences in modification presumably occur as a consequence of a solution condition-dependent conformational change in the anticodon loop.

In the crystal structure, the anticodon loop is found in a conformation very similar to that proposed by Fuller & Hodgson (1967) called the 3′-stacked conformation. Here, all but two of the anticodon loop nucleotides (Cm32 and U33) are stacked on the 3′ side of the loop (Robertus et al., 1974; Quigley & Rich, 1976; Holbrook et al., 1978). An alternate structure for the anticodon loop was also suggested in which the major stack occurs on the 5′ side of the loop, i.e., the

Table II: Comparison of Diffusivity and Modification Results[a]

solution condition					
Mg^{2+} (mM)	μ (M)	tRNA diffusivity	rel extent of modification: D16	G20	U33–Gm34
1	0.1	high	high	intermediate	high
1	0.25	low	low	intermediate	intermediate
10	0.1	high	intermediate	high	intermediate
10	0.25	high	low	high	low

[a] Comparison of the diffusion and chemical modification results obtained for $tRNA^{Phe}$ under identical solution conditions. The diffusivity results are from the upper left inset figure in Figure 3 while the modification results are from Table I and the lower panel in Figure 3.

5′-stacked conformation (Fuller & Hodgson, 1967; Woese, 1970). While it is clear that the 3′-stacked conformation obtains in the crystal structure, there is no consensus of opinion on which conformation exists in solution or, indeed, that firm evidence even exists for the occurrence of a 5′ stack. Results from most NMR and Y-base fluorescence studies are consistent with a 3′-stacked conformation, though Beardsley et al. (1970) present evidence that the anticodon loop can be quite flexible. A 5′-stacked conformation appears to be consistent with results from a number of oligonucleotide binding studies [for a review, see Cole & Crothers (1978)].

In the 3′-stacked conformation Gm34 is at the bottom of the stack and should be available for modification, while in the 5′ orientation Gm34 is in the middle of the stack. In the 5′ configuration the accessibility of this residue would be more limited, resulting in decreased reactivity with carbodiimide. It is possible that in 1 mM $MgCl_2$, 0.1 M ionic strength, the anticodon loop is in the 3′-stacked conformation, but in the 5′-stacked conformation at higher magnesium concentrations and ionic strengths. Another possibility is that the two conformations are in equilibrium, with the 3′-stack configuration being favored in 1 mM $MgCl_2$ at an ionic strength of 0.1 M. Raising either the magnesium concentration or the ionic strength could shift the equilibrium in favor of the 5′-stacked conformation. A similar change in the conformation of the anticodon loop has also been suggested by Urbanke & Maass (1978) to explain a relatively slow change in the Y-base fluorescence of yeast $tRNA^{Phe}$ in temperature-jump experiments.

The accessibility of U33 might be altered by yet another mechanism, one for which a clearly established precedent does exist. The recent elucidation of the crystal structure of the *E. coli* initiator tRNA, $tRNA_f^{Met}$, revealed a marked difference in the orientation of the structurally conserved U33 when compared with yeast $tRNA^{Phe}$ (Woo et al., 1980). The orientation of U33 in $tRNA_f^{Met}$ is almost opposite from that in $tRNA^{Phe}$, being directed out and away from the anticodon loop and into the surrounding solvent. Aside from this difference the anticodon bases in the two tRNAs are stacked in roughly similar fashion. The different orientation of U33 in the initiator species may be related to its unique function and caused by undefined structural features peculiar to this species. However, it is also possible that this base is highly mobile, having the ability to swivel in and out of the folded anticodon loop. Such a possibility has been suggested as a means of discriminating between A and P sites in the ribosome by $tRNA_f^{Met}$ or playing a role in the movement of elongator tRNAs from the A to P site (Woo et al., 1980). In the context of the present study the swiveling of this base could be influenced by the prevailing ionic conditions and thereby provide the basis for the observed chemical reactivity.

The modification of D16 and G20 in the D loop is sensitive to changes in ionic strength and magnesium concentration, but in different ways. The modification of D16 is largely dependent on the ionic strength, though in conditions of low salt there is some dependence on magnesium (Tables I and II). On the other hand, the modification of G20 appears to be quite independent of ionic strength but dependent on magnesium. In the crystal structre, D16 is highly exposed to solution. Therefore, it is to be expected that it will be modified by carbodiimide, though to a relatively low extent since dihydrouridine reacts slower with carbodiimide than the other reactive residues (Rhodes, 1975). As in the case of the anticodon loop, it seems likely that the differences in reactivity of D16 could be caused by a localized change in conformation that limits the accessibility of D16, possibly by burying it in the interior of the D loop. Such a change has not been suggested before but may well be possible because of the relative lack of constraint on the thermal motion of this base (Holbrook et al., 1978).

The modification of G20 seems to be wholly dependent on the magnesium concentration, with the higher extents of modification occurring in the 10 mM Mg^{2+} conditions. It is interesting to note that there are two magnesium binding sites in the D loop in the crystal structure and that one of the magnesiums is probably complexed directly to G20 (Teeter et al., 1980). It is possible that this site is fully occupied only in 10 mM $MgCl_2$ and that the additional binding causes G20 to become more available for carbodiimide reaction.

Also of interest is the recognition that the modification patterns observed in the current study are, with one or two exceptions, in good agreement with theoretical expectations of reactivity recently predicted for this tRNA (Lavery et al., 1980). In this work electrostatic potentials and steric accessibilities were calculated for the potentially reactive atoms of each base in the static crystal structure, assuming four site-specifically bound magnesium ions and attack by an electrophilic agent the size of the hydrogen atom. Of the guanine residues, G19, G20, and Gm34 were predicted to have good reactivity based on considerations of both accessibility and electronegativity. While G20 and Gm34 were modified in the present study, G19 was not. The lack of reactivity of this base is presumably due to its involvement in a tertiary bond with C56 (Figure 4); in this association the N1 atom of G19, the site of electrophilic attack by carbodiimide, participates in a hydrogen bond and would thus be shielded from reaction.

According to the theoretical study four U bases should be reactive to the model electrophilic agent, namely, D16, D17, U33, and U47. Three of these were modified in the present study, with D17 being the lone exception. From calculations D17 should be as accessible as D16 with only slightly lower electrostatic potential. A possible explanation for the absence of detectable reaction under all conditions evaluated may be screening by an additional magnesium ion now believed to be bound in this region (Teeter et al., 1980) but not considered in the theoretical study.

Changes in Diffusivity. The modification results like the diffusion measurements support the view that ion-dependent changes do, in fact, occur in the solution structure. Some of the effects described may reflect small, localized changes while others may be of a more global nature. However, it does not necessarily follow that structural changes which influence modification will also affect diffusivity and vice versa. Indeed, the lack of correlation between the diffusivity and modification results suggests that this is not the case. It is necessary therefore, that any explanation of the changes in diffusion or modification does not require a concomitant change in the other.

The diffusion coefficient is a hydrodynamic property and as such is effected not only by the size and shape of the tRNA itself but also by the size and shape of the shield of counterions and water molecules associated with it. Therefore, it is possible that changes in ion binding and hydration resulting from subtle, localized conformational changes could be responsible for the observed alterations in diffusivity. The change in the shape and size of the ion–water shell need not be large. Assuming a radius of gyration of 24 Å for tRNA (Pilz et al., 1970), a 14% increase in the diffusivity—the increase seen for $tRNA^{Phe}$—would require only a 1.6 Å increase in the radius of gyration. An increase of this order corresponds to the

addition of less than one layer of water molecules. Thus, a change in hydration and ionic composition could be a major contributor to the altered diffusion behavior.

The diffusion changes could also be caused by a dynamic change in structure as opposed to the static model just described. For example, it is possible that a change in the rate of breathing of the tRNA, say involving extension–contraction of helical stacks and/or rapid making and breaking of tertiary-level hydrogen bonds, could significantly affect the diffusivity. Such effects could be the basis for at least some of the changes observed in the laser light scattering studies.

Consistent with either model are results from an analysis of $tRNA^{Phe}$ conducted with S1 nuclease and RNase T_1 (Wrede et al., 1979). In this study no changes in the digestion patterns of yeast $tRNA^{Phe}$ were observed when Mg^{2+} and salt were varied over the ranges 0–10 mM and 0–0.1 M, respectively, conditions which encompass the ionic conditions where the diffusion changes were observed. While these negative results can also be used to support the conclusion that large, metastable changes are not involved, like the modification results they do not permit us to rule on the alternative models proposed. Both the enzymatic and chemical probes may be thermodynamically and/or kinetically disadvantaged. Although the results of the present study do not permit us to distinguish between the models proposed, it is possible that high-resolution proton NMR, especially the time-resolved, low-field Fourier transform technique of Johnston & Redfield (1977), can. Such analyses are being planned.

The results described here, and the models proposed to explain them, could be physiologically relevant. The conformational changes detected in both the diffusion and chemical modification studies occur in the physiological range of pH, ionic strength, and magnesium concentration. Small changes in local ionic conditions in the cell, say in Mg^{2+} flux, could cause changes in tRNA conformation that might be functionally important in the recognition and binding processes in which tRNA participates.

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Phospholipid Vesicle Aggregation: Effect of Monovalent and Divalent Ions†

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ABSTRACT: A study on cation-induced aggregation of unilamellar phospholipid vesicles was made by measuring the turbidity of vesicle suspensions. As the cation concentration was increased, the degree of aggregation of acidic phospholipid vesicles increased. The concentration at which the sharpest increase in turbidity was observed was defined as the "threshold" concentration. The threshold concentrations of various divalent cations for aggregation of phosphatidylserine vesicles in 0.1 M NaCl, pH 7.0, were 0.65 mM Mn^{2+}, 0.8 mM Ba^{2+}, 1.0 mM Ca^{2+}, 1.2 mM Sr^{2+}, and 4.5 mM Mg^{2+}. This order of threshold concentrations was the same for vesicles composed of mixtures of phosphatidylserine and phosphatidylcholine. A decrease in the monovalent ionic strength of the vesicle suspension solutions raised the threshold concentrations for Mn^{2+} and Ca^{2+}. The order of effectiveness for monovalent cations to cause phosphatidylserine vesicle aggregation was $H^+ > Na^+ > Li^+ > K^+ > TMA^+$ (tetramethylammonium). The effect of pH on phosphatidylserine vesicle aggregation was studied in the presence of Na^+. The threshold pH for vesicle aggregation was about 2.6 in 100 mM NaCl. An attempt was made to explain vesicle aggregation in terms of surface potential, surface charge densities of the membranes, and the electrostatic repulsive interaction energy between two vesicles. It was proposed that the slope of the total interaction energy (repulsive interaction) at the Debye distance is related to the degree of massive aggregation occurred among phosphatidylserine vesicles. Knowing the intrinsic binding constants of Ca^{2+} (30 M^{-1}) and Na^+ (0.6 M^{-1}) to a phosphatidylserine membrane and assuming that the slope of the interaction energy of two interacting membranes is the same at the Debye distance at the observed threshold concentrations of ions for vesicle aggregation, we calculated the binding constants of other metal ions: Mn^{2+} = 49 M^{-1}, Ba^{2+} = 37 M^{-1}, Sr^{2+} = 25 M^{-1}, Mg^{2+} = 10 M^{-1}, Li^+ = 0.4 M^{-1}, K^+ = 0.2 M^{-1}, and H^+ = 100 M^{-1}.

For elucidation of the molecular mechanism of membrane fusion, which is involved in many biological cellular processes (Poste & Allison, 1973), membrane fusion studies using lipid model membrane systems have recently been made by a number of laboratories (Prestegard & Fellmeth, 1974; Papahadjopoulos et al., 1974; Breisblatt & Ohki, 1975; Koter et al., 1978; Ingolia & Koshland, 1978; Liao & Prestegard, 1979; Wilschut & Papahadjopoulos, 1979). The close contact of two membranes is considered to be an initial step and a necessary condition for two membranes to fuse. Thus, cell membrane aggregation studies should be relevant for understanding conditions and control of membrane fusion reactions. For vesicle aggregation studies, turbidity (Chong & Colbow, 1976) and light scattering (Lansman & Haynes, 1975; Day et al., 1977) methods have been frequently utilized. Although the interpretation of turbidity is not as clear as that of light scattering, turbidity studies have provided some useful information for elucidation of the mechanisms of aggregation, fusion, and membrane structural alterations of vesicle membranes.

The effect of temperature on the turbidity of lipid vesicle suspensions has been studied in relation to the phenomenon of the gel to liquid-crystalline phase transition (Yi & MacDonald, 1973; Martin & MacDonald, 1976; Peterson & Chan, 1978; Kremer & Wiersema, 1977). It is well documented that acidic phospholipid vesicles aggregate in the presence of cations whose "threshold" concentration depends upon the membrane composition and concentrations of ionic species in the suspension solution. The flocculation of sonicated phosphatidylserine vesicles due to high concentrations of NaCl and KCl and millimolar concentrations of $CaCl_2$ and $MgCl_2$ has been observed by several investigators (Abramson et al., 1964; Hauser & Phillips, 1973; Papahadjopoulos et al., 1975). The irreversible changes in flocculation have been interpreted as evidence for membrane fusion and structural alterations of vesicle membranes (Papahadjopoulos et al., 1974; Chong & Colbow, 1976). However, further systematic studies of phospholipid vesicle aggregation are necessary to understand ionic and membranous factors that control membrane fusion reactions. These factors include the surface charge density

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