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Proton Nuclear Magnetic Resonance Study of the Effect of pH on tRNA Structure[†]

M. Steinmetz-Kayne,* R. Benigno, and N. R. Kallenbach

ABSTRACT: The low-field 220-MHz proton nuclear magnetic resonance (NMR) spectra of four tRNA molecules, *Escherichia coli* tRNA^{Phe}, tRNA^{Val}, and tRNA^{Met}, and yeast tRNA^{Phe}, at neutral and mildly acidic pH are compared. We find a net increase in the number of resonances contributing to the -9.9-ppm peak (downfield from sodium 4,4-dimethyl-4-silapentanesulfonate) in three of these tRNAs at pH 6, while tRNA^{Met} does not clearly exhibit this behavior. The increase in intensity at this resonance position is half-completed at pH 6.2 in the case of yeast tRNA^{Phe}. An alteration at the 5'-phosphate terminus is not involved, since removal of the ter-

minal phosphate does not affect the gain in intensity at -9.9 ppm. Based on a survey of the tertiary interactions in the four molecules, assuming that they possess tertiary structures like that of yeast tRNA^{Phe} at neutral pH, we tentatively attribute this altered resonance in *E. coli* and yeast tRNA^{Phe} to the protonation of the N₃ of the adenine residue at position 9 which results in the stabilization of the tertiary triple A₂₃·U₁₂·A₉. This interpretation is supported by model studies on the low-field proton NMR spectrum of A_N oligomers at acid pH, which reveal an exchanging proton resonance at -9.4 ppm if the chain length *N* ≥ 6.

Since their original implication in protein biosynthesis, transfer RNA molecules have been found to function in highly diverse roles within the cell (Littauer and Inouye, 1973). The variety of interactions demonstrated by these molecules in protein synthesis suggests that more than one stable structure may exist in vivo. It would in fact be remarkable to find tRNA molecules binding to aminoacyl-tRNA synthetases, T-factors, codon triplets, and ribosomes without structural alteration. The determination of a detailed structure for yeast tRNA^{Phe} from diffraction analysis of crystalline molecules (review, Sussman and Kim, 1976) has stimulated attempts both to define the precise conformation of tRNA molecules in solution as well as to determine the nature of the conformational changes that arise as a function of the solution conditions. Observed changes in the overall absorbance or circular dichroism properties of individual tRNA molecules as well as unfractionated tRNA mixtures (Riesner et al., 1969; Römer et al., 1970; Goldstein et al., 1972; Cole et al., 1972) indicate that the in vitro structure of tRNA is highly sensitive to ionic strength, divalent and monovalent cations, temperature, pH, and solvent, to a degree not observed in single- or double-stranded RNA structures. In situ conformational probes, such as the 4-thiouridine residue present in certain *Escherichia coli* tRNAs (Delaney et al.,

1974) or the Y base in yeast tRNA^{Phe} (Beardsley et al., 1970), reveal similar alterations in conformation, as do the tritium exchange rates of hydrogen-bonded protons in tRNA (Goldstein et al., 1972) and the hydrodynamic properties of the molecule (Fresco et al., 1966; Olson et al., 1976).

The most detailed attempted comparisons of the solution and crystal structure of tRNA have been based upon high-resolution proton nuclear magnetic resonance studies of the hydrogen bonds in a number of specific tRNAs. The key observations that the ring protons of guanine (N₁H) and uracil (N₃H) exchange slowly when they participate in hydrogen-bonded base pairing of tRNA (Englander and Englander, 1972) and that their nuclear magnetic resonance frequency is sufficiently removed from that of the water protons to permit their measurement directly in H₂O (Kearns et al., 1971a) have led to an extensive series of investigations on the solution structure of tRNA (Kearns and Shulman, 1974; Crothers et al., 1974; Daniel and Cohn, 1975; Reid and Robillard, 1975; Robillard et al., 1976). With the recent finding that tertiary as well as secondary hydrogen bond resonances can be detected in the low-field spectral region (Reid et al., 1975), and that the total number of resonances is in fair agreement with that anticipated from the x-ray models (Sussman and Kim, 1976), it seems reasonable to anticipate that a complete assignment of all secondary and tertiary bond resonances will emerge in the near future.

If the conclusion can be accepted that the structure of tRNA in solution, in the presence of sufficient Mg²⁺, at neutral pH and temperature below 37 °C, corresponds reasonably well with the x-ray crystallographic structure of yeast tRNA^{Phe}

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(Daniel and Cohn, 1975; Robillard et al., 1976), then the question of changes occurring in this fundamental conformation can be approached. Recently, a number of proton magnetic resonance investigations concerned with alterations of tRNA structure in response to metal binding (Reid and Robillard, 1975), dye binding (Jones and Kearns, 1975), and aminoacylation (Wong et al., 1973), as well as the more extensive native-to-denatured transitions (Rordorf et al., 1976), have appeared.

In this report we are concerned with the effects of pH on the structure of tRNA molecules in solution. The subject of the structure and function of tRNA at acid pH has received attention since it has been observed that the binding of tRNAs to their cognate aminoacyl-tRNA synthetases is enhanced at pH 5.5 relative to pH 7 (Yarus and Berg, 1967; Lam and Schimmel, 1975). Based on relaxation kinetic analysis, conformational changes have been reported at acid pH, for *E. coli* tRNA^{fMet} and tRNA^{Tyr} (Bina-Stein and Crothers, 1974, 1975). Lynch and Schimmel (1974) studied the fluorescence of a probe attached to the amino acid on *E. coli* tRNA^{Ile}. They found that certain bases in tRNA exhibit abnormally high pKs in the presence of low salt and that protonation of these sites leads to stabilization of an acid form. A dramatic alteration in Mg²⁺ binding to this tRNA as a function of pH occurred.

Earlier NMR studies of *E. coli* and yeast tRNA^{Phe} (Kearns et al., 1971b; Wong et al., 1972) indicate the presence of additional resonances in the -9 to -11 ppm region of the spectrum at acid pH. Solvent perturbation studies suggest that the additional resonances might be associated with tertiary structure and are exceptionally sensitive to the solvent conditions. In the present study we have carried out a high-resolution NMR study of the resonances in the low-field region, -9 ppm or more downfield from DSS.¹

We report here a comparison of the low-field proton NMR spectra of four tRNA molecules, *E. coli* tRNA^{Phe}, tRNA^{Val}, and tRNA^{Met}, and yeast tRNA^{Phe}, at neutral and mildly acidic pH. We find that the resonances in this region appear to be associated with protons of the tertiary structure in these molecules and that they are more sensitive to variations in pH and temperature than the predominantly secondary structural resonances below -11 ppm.

Materials and Methods

Yeast tRNA^{Phe} and *E. coli* tRNA^{Val} were purchased from Boehringer Mannheim GmbH. The sample of *E. coli* tRNA^{fMet} was obtained from Dr. Mildred Cohn (for preparation refer to Daniel and Cohn, 1975). *E. coli* tRNA^{Phe} was isolated from crude *E. coli* B tRNA (Schwarz/Mann) by aminoacylation and chromatography on BD-cellulose (Roy et al., 1967). All tRNAs had an amino acid accepting capacity of 1000 pmol/A₂₆₀ unit or greater.

Sources of chromatographic materials used in this study are as follows: Sephadex G-25 (Pharmacia); silicic acid (Fischer); BD-cellulose (Schwarz/Mann); RPC-5 (Pearson et al., 1971) was prepared using plascon support and androgen 464 kindly supplied by Dr. G. D. Novelli, Oak Ridge, Tenn.

Preparation of Oligo(rA)₅ and Oligo(rA)₆. Limited hy-

drolysis of poly(A) (Sigma) was carried out according to the procedure of Eichhorn and Butzow (1965). The 5'-terminal phosphate was removed by treatment with bacterial alkaline phosphatase (Worthington) and polymers of various chain lengths were separated by RPC-5 chromatography (Egan and Kelmers, 1974). Separation was achieved in 0.1 M Tris-HCl (pH 7.4) and 0.001 M EDTA employing a gradient of 0.01 to 2.0 M NaCl. The chain lengths of the isolated polymers were determined by thin-layer chromatography and compared to oligo(A) chains of known length obtained from Collaborative Research.

Preparation of 5'-OH Yeast tRNA^{Phe}. Prior to incubation with enzyme, yeast tRNA^{Phe} was treated to remove tightly bound divalent metal ions (Bernardi and Cantoni, 1969) since failure to do so was found to restrict digestion. Bacterial alkaline phosphatase (BAPF) was purchased from Worthington. Residual RNase was inactivated by heating the enzyme preparation for 15 min at 85 °C in 0.01 M MgCl₂ and 0.01 M Tris-HCl (pH 7.8). Yeast tRNA^{Phe} (10 mg) and alkaline phosphatase (100 units) were incubated for 10 min at 45 °C (Stern et al., 1969), and the reaction was followed by measuring the amount of phosphate released as a function of time (Ames and Dubin, 1960). After 10 min, approximately 1.2 mol of phosphate was released per mol of tRNA. Enzyme and free phosphate were removed by passage over a silicic acid-Sephadex G-25 column (Miller et al., 1970) and the tRNA was recovered by ethanol precipitation. The tRNA was suspended in buffer, and dialyzed extensively.

Preparation of Aminoacyl-tRNA. [³H]Val-tRNA^{Val} was prepared according to the method of Lagerkvist and Waldestrom (1964) using a partially purified mixture of *E. coli* tRNA synthetases (Pillinger et al., 1969). Aminoacylation of yeast tRNA^{Phe} was achieved using purified yeast phenylalanyl-tRNA synthetase prepared according to the method of Schmidt et al. (1971) utilizing toluene lysis at -20 °C (Fell et al., 1974). tRNA was recovered by ethanol precipitation, suspended in 0.01 M cacodylate buffer (pH 5.7), 0.01 M MgCl₂, and 0.1 M NaCl, and dialyzed for 3 h. NMR experiments were conducted immediately after dialysis. Following recording of the spectrum for several hours, an aliquot of the sample was precipitated and counted, and found to be 80% aminoacylated.

Preparation of NMR Samples. tRNA samples (5-10 mg) were suspended in approximately 0.15 mL of buffer. The sample was then dialyzed for 12 to 24 h in a flow dialysis chamber. pH titrations were performed by dialyzing the sample in each case against the appropriate buffer. *E. coli* tRNAs were routinely shielded from the light so as to prevent photoreaction of 4-thiouridine (Favre et al., 1969).

Nuclear Magnetic Resonance Measurements. Nuclear magnetic resonance spectra were recorded on a Varian HR 220-MHz spectrophotometer operating in the CW mode. Signals were averaged to improve spectral signal-to-noise ratios with a Varian 6201 computer. Sample temperatures were controlled within 0.4 °C by means of a Varian variable-temperature accessory. Chemical shifts are reported in terms of their shift in parts per million downfield from the position of the methyl resonance of DSS. Two different standards were used to determine the number of exchangeable protons per tRNA molecule. The integration of the yeast tRNA^{Phe} spectra was achieved by assigning a value of four to the resonance at -13.7 ppm (Reid and Robillard, 1975) while for the *E. coli* tRNAs, a value of one was assigned to the -14.9-ppm peak (Reid et al., 1975; Daniel and Cohn, 1975).

Absorption and CD Spectra. Optical changes in yeast

¹ Abbreviations used are: DSS, sodium 4,4-dimethyl-4-silapentane-sulfonate; A₂₆₀ unit, the quantity of tRNA contained in 1 mL of solution which has an absorbance of 1 at 260 nm when measured in a 1-cm path length cell; CD, circular dichroism; BD-cellulose, benzoyldiethylaminoethylcellulose; s⁴U, 4-thiouridine; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

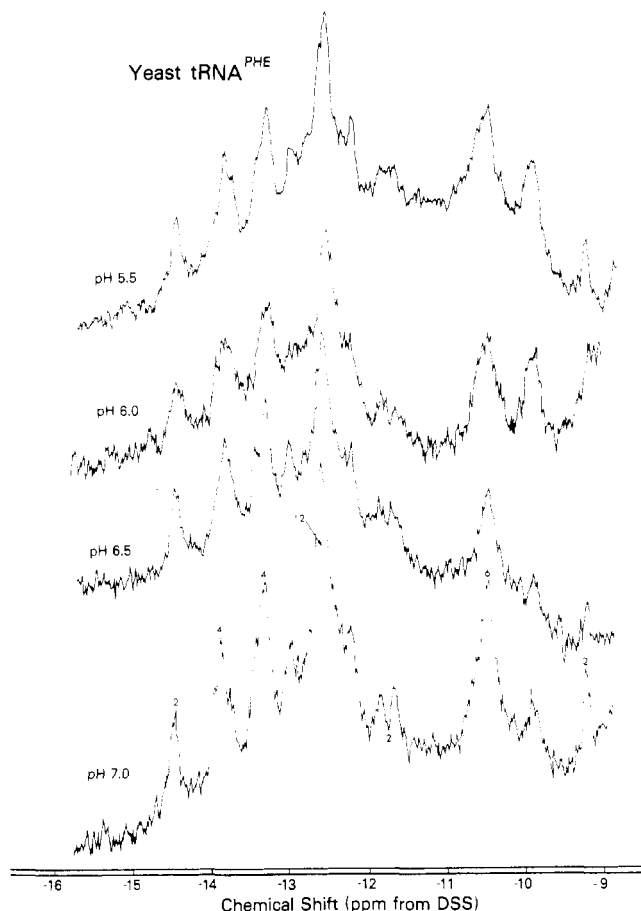


FIGURE 1: Low-field proton NMR spectra of yeast $tRNA^{Phe}$ as a function of pH. Solution conditions: 1.5 mM yeast $tRNA^{Phe}$, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM sodium cacodylate buffer, 35 °C. The numbers above the pH 7.0 spectra indicate the integrated spectral area of each peak.

$tRNA^{Phe}$ between pH 7 and 5.5 were measured as follows: equal volumes of a concentrated stock tRNA solution were diluted by weighing into two matched cuvettes containing buffers to yield equal volumes of solution at pH 7 and 5.5 at a final concentration of 1.2 A_{260} units. The two solutions were then scanned on a Cary 118 double beam spectrophotometer to produce the curve reported. The data were taken manually at a number of wavelengths on a Cary 16 instrument for comparison. These solutions were used to record the CD spectra reported in Figure 7 using a Durrum Jasco J10 instrument. All spectra were run at room temperature. The $[\theta]$ values were calculated using the value of $A_m = 5.02 \times 10^5$ for the extinction coefficient of yeast $tRNA^{Phe}$ (Blum et al., 1972).

Results

The low-field 220-MHz proton NMR spectra of yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Phe}$, $tRNA^{fMet_1}$, and $tRNA^{Val_1}$, at neutral and acid pH, are shown in Figures 1–4. We have investigated an extended region of the spectra from –9 to –15 ppm. The –11- to –15-ppm region includes the ring N_1H of G and N_3H of U when they participate in G·C and A·U Watson-Crick base pairing. The position of each ring proton in the secondary structure can be estimated from the intrinsic positions for the two resonances, i.e., N_1H of G in G·C resonates at approximately –13.6 ppm from DSS, and N_3H of U in A·U at –14.6 ppm (Kearns and Shulman, 1974; Kan et al., 1974) and the deshielding effect exerted by the ring

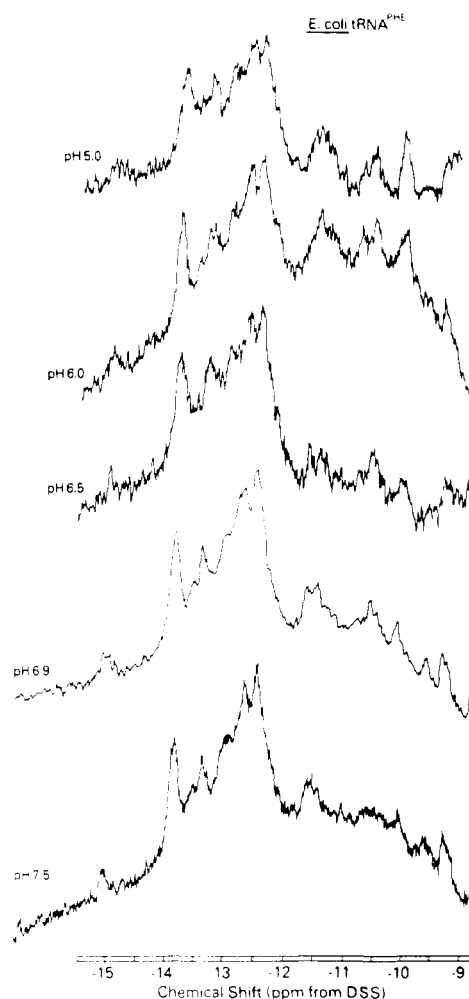


FIGURE 2: Low-field proton NMR spectra of *E. coli* $tRNA^{Phe}$ as a function of pH. Solution conditions: 2 mM *E. coli* $tRNA^{Phe}$, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM sodium cacodylate buffer, 30 °C.

currents of nearest neighbor bases in the helix. It has been found that a number of tertiary structural base pairs also have protons resonating in this region, including the $s^4U_8 \cdot A_{14}$ pair in a number of *E. coli* tRNA species (Daniel and Cohn, 1975; Reid et al., 1975). As proposed by Reid and coworkers (1975) and in this report, the –9- to –10-ppm region may include tertiary resonances of exocyclic amino hydrogens which are hydrogen bonded to ring nitrogens (Figure 5A,B).

Yeast $tRNA^{Phe}$. The low-field spectrum of yeast $tRNA^{Phe}$ at neutral and acid pH at 35 °C is shown in Figure 1. We have calculated, from the intensities indicated in the lowest panel of Figure 1, a total of 34 protons in the spectrum with a minimum of 24 protons between –11 and –15 ppm. For comparison, the higher resolution 360-MHz NMR spectrum obtained by Reid and Robillard (1975) includes 26 protons in the latter region.

When the spectrum at pH 7.0 is compared to that obtained at acid pH, the –11- to –15-ppm region shows no major change in either intensity or position, at the resolution we have obtained. Clearly there may be alterations but investigation of these will require improved resolution.

At pH 7, the –9- to –11-ppm region contains 4 resonances at –8.9, –9.2, –9.9, and –10.5 ppm with relative intensities of 1:2:1:6, respectively (cf. Figure 10). At acid pH, the intensity at –9.9 ppm exhibits an increase in intensity corresponding to two protons while the intensity of other resonances in this

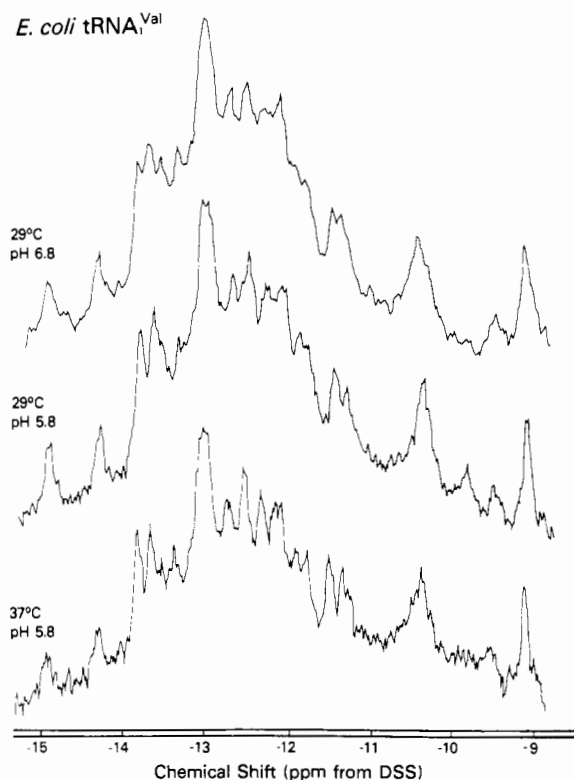


FIGURE 3: Low-field NMR spectra of *E. coli* tRNA^{Val}₁ as a function of pH. Solution conditions: the same as given in Figure 2.

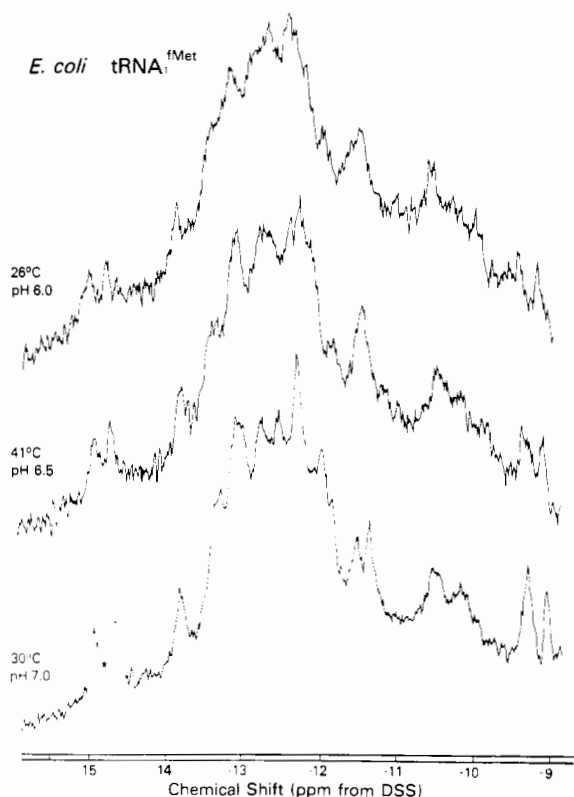


FIGURE 4: Low-field proton NMR spectra of *E. coli* tRNA^{fMet}₁ as a function of pH. Solution conditions: 2 mM *E. coli* tRNA^{fMet}₁, 100 mM NaCl, 10 mM MgCl₂, 10 mM sodium cacodylate buffer.

region is unaltered. A graph of the relative intensity of the resonance at -9.9 ppm at different pH values is shown in Figure 6. The resonance undergoes an abrupt increase in in-

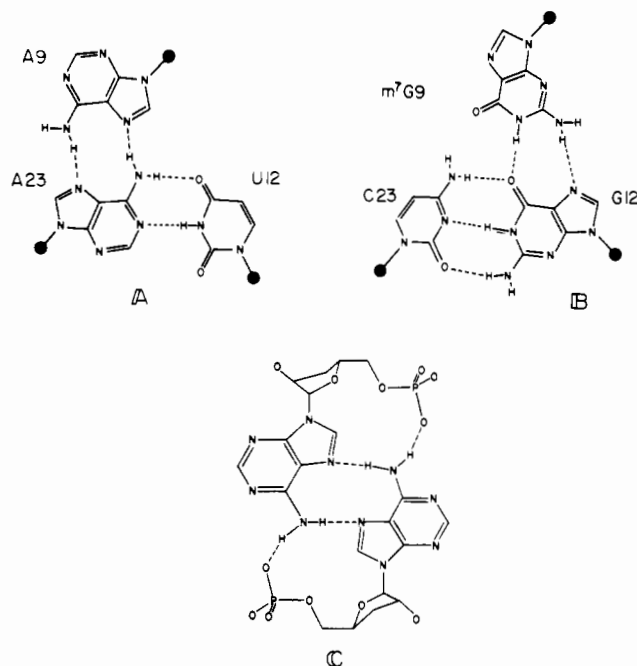


FIGURE 5: Comparison of tertiary structure base pairing and acid oligo(A) interactions: (A) A9-A23-U12 tertiary bond of tRNA^{Phe}; (B) G9-C23-G12 postulated for *E. coli* tRNA^{fMet}₁ (Kim et al., 1974); (C) crystal structure of poly(A) (Rich et al., 1961).

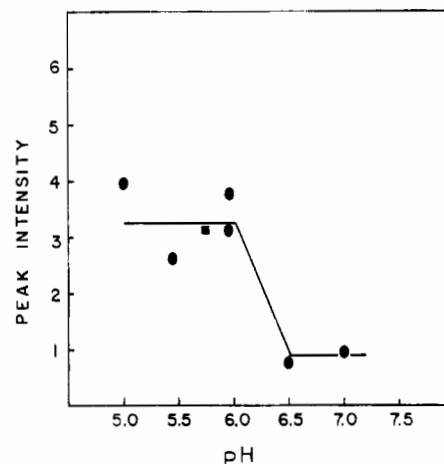


FIGURE 6: Intensity of the -9.9-ppm resonance as a function of pH. Solution conditions: 1.5 mM yeast tRNA^{Phe}, 100 mM NaCl, 10 mM MgCl₂, (●) 10 mM sodium cacodylate, (■) 10 mM sodium phosphate, 35°C.

tensity between pH 6.5 and 6.0, the apparent midpoint being at pH 6.25. The data indicate that the protonation of one or more residues on yeast tRNA^{Phe} results in the stabilization of two additional hydrogen-bonded protons.

E. coli tRNA^{Phe}. The spectrum of *E. coli* tRNA^{Phe} follows a parallel course (Figure 2) in response to pH. The -9- to -11-ppm region of *E. coli* tRNA^{Phe} spectrum at pH 6.9 (Figure 2) is different from that of yeast tRNA^{Phe}, having resonances at -9.1, -9.6, and -9.9 ppm with intensities of 2:1:1, respectively. An apparent increase in intensity at -9.9 ppm occurs at acid pH. As the pH is lowered from 6.5 to 6.0, the number of protons contributing to the -9.9-ppm peak increases to a value of 2.5 ± 0.5 . It is difficult to calculate an accurate intensity value since measurements are based on the intensity of the -14.9-ppm peak. However, the data indicate that although yeast and *E. coli* tRNA differ considerably in

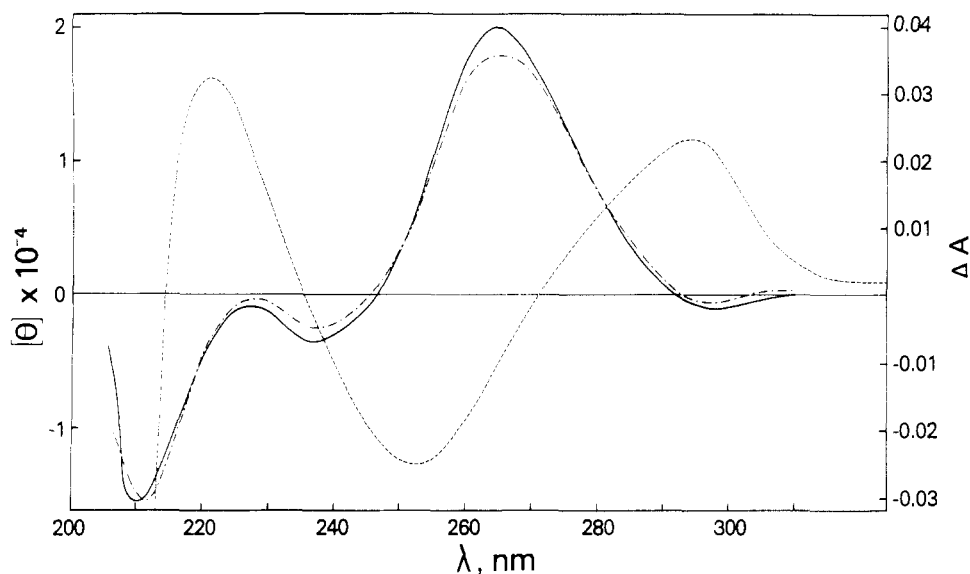


FIGURE 7: Differential absorbance and CD spectra of yeast tRNA^{Phe} at neutral vs. acid pH. The CD spectra of 1.4 μ M yeast tRNA^{Phe} in 500 μ M NaCl, 50 μ M MgCl₂, and 670 μ M Na⁺-K⁺ phosphate buffer was measured at pH 5.5 (---) and pH 7.0 (—). The same solutions were used to record the UV difference spectrum (- · -).

primary structure, the two molecules undergo a very similar structural response to pH change.

E. coli tRNA^{Val}₁. At pH 6.8, the -9- to -11-ppm region of *E. coli* tRNA^{Val}₁ is similar in respect to both position and intensity to that of *E. coli* tRNA^{Phe}, exhibiting resonances at -9.1 and -9.6 ppm in a ratio of 2:1 (Figure 3). Under our experimental conditions, no resonance at -9.9 ppm is observed until the pH is lowered to pH 5.8, whereupon a resonance appears with an intensity of one. This resonance is exceptionally labile, since increasing the temperature from 29 to 37 °C results in a drastic broadening of this peak (Figure 3).

E. coli tRNA^{Met}₁. In the -9- to -11-ppm region, the spectrum of *E. coli* tRNA^{Met}₁ at pH 7.0 (Figure 4) resembles that of yeast tRNA^{Phe} having resonances at -9.1 and -9.4 in the ratio of 1:2. tRNA^{Met}₁ does not exhibit any obvious increase in the -10.5- to -9.5-ppm region as the pH is lowered from 7.0 to 6.0 although the spectrum is not sufficiently clear to exclude a change completely.

Optical Changes in Yeast tRNA^{Phe} between pH 7 and pH 5.5. The acid-induced resonances at -9.9 ppm may result indirectly from either a pH-induced slowing of the exchange rate of an otherwise rapidly exchanging proton (e.g., N₁H of G) or directly from protonation of one or more base residues that stabilize hydrogen bonds in the tRNA. To distinguish between these two possibilities, we have examined the spectral properties of yeast tRNA^{Phe} as a function of pH. We have verified that the yeast tRNA^{Phe}, for example, undergoes a change in ultraviolet (UV) absorption and in CD between pH 7 and 5.5 in our solvent conditions (Figure 7). The differential absorbance curve is consistent in form with protonation of an A residue ($pK = 3.5$), and less so with that of a C residue ($pK = 4.2$), based on the known spectral changes associated with protonating these bases. Both Bina-Stein and Crothers (1974, 1975) and Lynch and Schimmel (1974) have reported optical spectral changes in tRNA molecules over this range of pH.

Exchanging Protons in the Low-Field Proton NMR Spectrum of Acid Oligo(A). The resonances in the -9- to -11-ppm region have tentatively been assigned to either ring N-H protons associated with nonstandard base pairs or to the exocyclic amino protons of A, G, or C when hydrogen bonded to ring nitrogens (Wong et al., 1972; Reid et al., 1975). The x-ray

crystal structure of yeast tRNA^{Phe} reveals the presence of tertiary bonds involving structures of the latter type (Figure 5A) (Kim et al., 1974). The assignment of the HN-H...N protons to the -9- to -11-ppm region is consistent with the recent finding that the HN-H...O proton in the duplex d-CpG(pCpG)_n is located at ~8.4 ppm (Patel and Canuel, 1976); the exocyclic amino proton when hydrogen bonded to a ring nitrogen is expected to undergo a larger downfield shift than that observed when it is bound to oxygen (Katz and Penman, 1966).

In order to clarify the identity of the resonances of protons in the -9- to -11-ppm region, we have carried out a preliminary study of the low-field NMR spectrum of acid oligo(A)s under conditions favoring helix formation (Eigen and Pörschke, 1970).

The acid (A) helical structure (illustrated in Figure 5C) apparently does not directly involve the N₁H proton in an intramolecular hydrogen bond (Rich et al., 1961). Instead, both exocyclic amino hydrogens are bonded, one to N₇ of the associated adenine ring, the other to a phosphate oxygen acceptor. Since pairing of the exocyclic N-H to N₇ is postulated in yeast tRNA^{Phe} (Kim et al., 1974), adenylate oligomers were prepared and their proton NMR spectra in H₂O were measured at pH 4.2. These conditions favor stable double helices of oligo(A_H⁺·A_H⁺) (Eigen and Pörschke, 1970). Figure 8 shows the low-field region of the proton NMR spectrum for oligo(A₅) and -(A₆) at 3 °C and pH 4.2. A resonance attributable to the exchanging proton appears at -9.4 ppm in the spectrum of oligo(A₆), but not in that of oligo(A₅). If one assumes that the coordinates of the acid poly(A) helix apply to the oligomeric helix, and that protonated A has similar shielding properties to unprotonated adenine, then the shielding exerted by the ring currents of adjacent bases amounts to +1.2 ppm using the calculated value of Giessner-Pretre et al. (1976). This implies that the intrinsic unshielded resonance of the proton—taking it to represent the exocyclic N-H·N₇ hydrogen—would be about -10.6 ppm.

Proton NMR Spectrum, at Neutral and Acid pH, of Yeast tRNA^{Phe} with 5'-Phosphate Removed. The results of the foregoing experiments indicate that the appearance of additional resonances at -9.9 ppm at acid pH involves the pro-

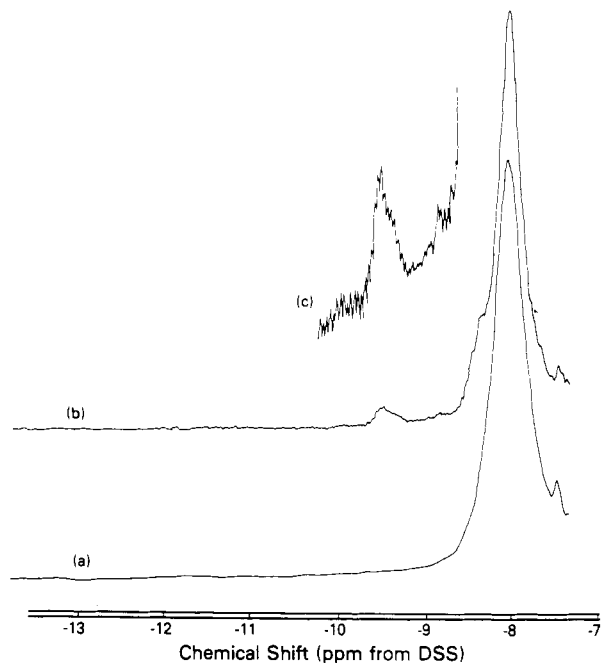


FIGURE 8: Low-field proton NMR spectrum of acid oligo(A)₅ and -(A)₆. NMR spectrum of ~10 mM oligo(A)₅ (a) and oligo(A)₆ (b) in 0.1 M deuteriomalonate (pH 4.33). The NMR spectrum (c) is an enlargement of the -8- to -10-ppm region of oligo(A)₆.

tonation of certain nucleotide residues which result in the formation and/or stabilization of hydrogen-bonded structures possibly of the HNH...N type. In order to further characterize the transition, we considered the group or groups of tRNA capable of such protonation.

Previous studies have shown that monomeric A and C have salt-independent pK_H values of 3.5 and 4.2, respectively (Steiner and Beers, 1961), while the protonation of acid poly(A) occurs at $pK_H \approx 5.9$ in 0.1 M KCl (Rich et al., 1961). Interestingly, the pK_H of oligomeric model compounds, as well as tRNA (Lynch and Schimmel, 1974), is greatly depressed in the presence of divalent metal ions. Since the NMR studies, described above, were conducted in the presence of saturating amounts of divalent cation (Mg^{2+}), the observed pH range for the intensity increase at -9.9 ppm appears to be well above that observed for either the monomeric or oligomeric structures previously studied.

One obvious residue in tRNA with a pK_H of 6.3 is the 5'-terminal phosphate (Gueron and Shulman, 1975). The following experiment was undertaken to determine if the removal of the 5'-terminal phosphate resulted in the elimination of the additional -9.9-ppm resonances.

A sample of yeast tRNA was digested with bacterial alkaline phosphatase under the conditions described under Materials and Methods. The digestion leads to formation of inorganic phosphate in concentrations stoichiometric with the amount of tRNA added. It is worth noting that the removal of the terminal phosphate could only be achieved under conditions where the tRNA structure was destabilized (Hilbers et al., 1973).

The low-field proton NMR spectrum of dephosphorylated yeast tRNA^{Phe} at pH 6.5 and 6.0 is shown in Figure 9. Despite some loss in resolution, the increase in intensity of the -9.9-ppm resonance is evident. Therefore, the 5'-phosphate is not involved in the behavior observed.

Thermal Stability of the -9.9-ppm Resonance in Yeast tRNA^{Phe} and *E. coli* tRNA^{Phe}. The exceptional lability of the

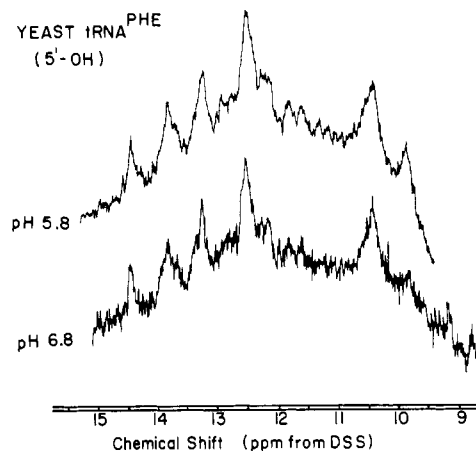


FIGURE 9: Low-field proton NMR spectrum of 5'-OH yeast tRNA^{Phe}. The solution conditions are the same as in Figure 1.

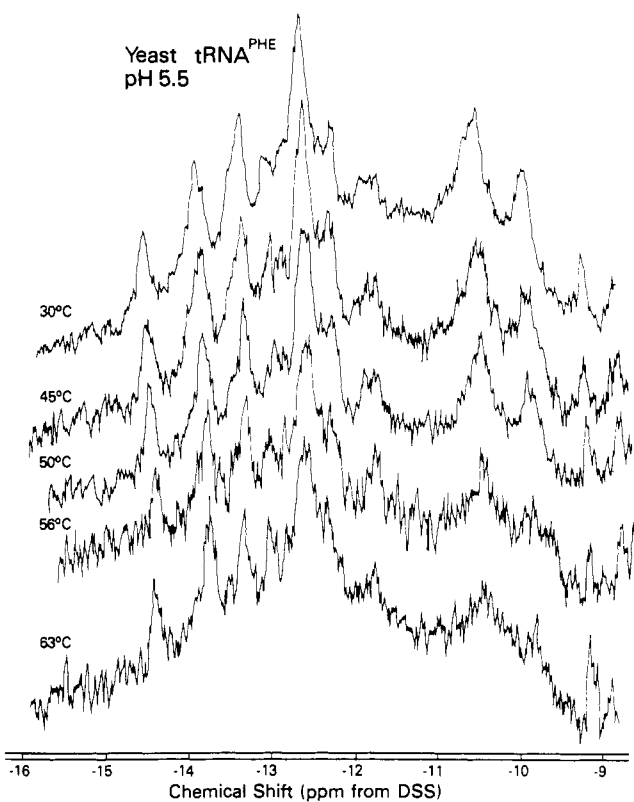


FIGURE 10: NMR spectrum of yeast tRNA^{Phe} (pH 5.5) as a function of temperature. Solution conditions: 1.5 mM yeast tRNA^{Phe}, 100 mM NaCl, 10 mM $MgCl_2$, and 10 mM sodium cacodylate buffer (pH 5.5).

-9.9-ppm resonance in the spectrum of *E. coli* tRNA₁^{Val}, at pH 7.0 and 5.8, has already been pointed out. A comparison of the yeast tRNA^{Phe} NMR spectra at pH 5.5 (Figure 10) and 7.0 (Figure 11) as a function of temperature shows several differences in the melting profiles of the two samples. At pH 7.0, there is a distinct broadening of the resonance at -10.5 ppm, and two resonances, located at -14.4 and -11.7 ppm, decrease in intensity, as the temperature is raised to 56 °C.

At pH 5.5 and 56 °C, there is considerable broadening of the -10.5- and -11.7-ppm resonances, but no intensity has been lost from the -14.4-ppm peak for example. At 63 °C, the -9.9-ppm region still shows a resonance corresponding to one proton. Since broadening and disappearance of the resonances are caused by a decrease in the lifetime of the proton in the

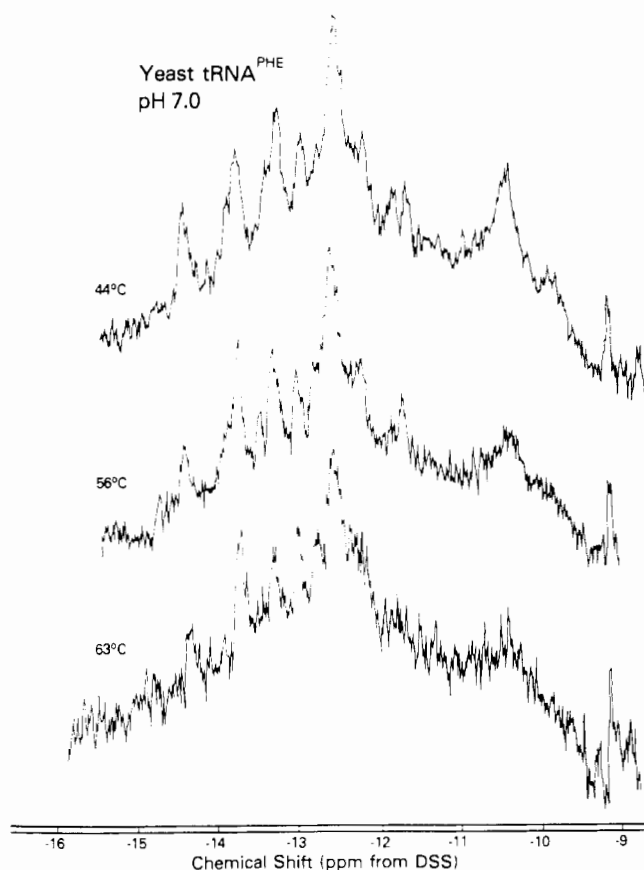


FIGURE 11: NMR spectrum of yeast tRNA^{Phe} (pH 7.0) as a function of temperature. Solution conditions: 1.5 mM yeast tRNA^{Phe}, 100 mM NaCl, 10 mM MgCl₂, and 10 mM sodium cacodylate (pH 7.0).

hydrogen-bonded structure, the apparent retention of the -14.4 - and -9.9 -ppm resonances at 63°C at pH 5.5 compared to that at pH 7.0 points to an apparent stabilization of at least some features of the yeast tRNA^{Phe} structure at acid pH. The stabilization is apparently the result of protonation of various residues in the tRNA structure.

The thermal profiles of *E. coli* tRNA^{Phe} at pH 7.0 are roughly similar (cf. Figures 11 and 12). In accord with previous findings (Kearns et al., 1971b), the pH 7.0 spectrum of *E. coli* tRNA^{Phe} shows considerable broadening in the -10 - to -12 -ppm region at 56°C , while the -12 - to -14 -ppm region still exhibits sharply defined resonances at this temperature. In contrast, at pH 5.5, the melting profile of *E. coli* tRNA^{Phe} (Figure 13) shows significant broadening of the entire -9 - to -15 -ppm region at 48°C . This is considerably lower than that observed at pH 7.0 so that the structure of this particular tRNA may in fact be more thermolabile at pH 5.5 than at pH 7.0. Higher resolution spectra would be required to establish this clearly.

Low-Field NMR Spectrum of Aminoacylated tRNA. Since the -9.9 -ppm resonances at neutral and acid pH are very sensitive to changes in the physical environment, we examined the effect of chemical alteration of the environment, i.e., aminoacylation, on the -9.9 -ppm resonances.

The low-field proton NMR of valine tRNA^{Val} is shown in Figure 14. This spectrum was measured at 30°C in order to permit detection of the -9.9 -ppm resonance in this molecule. It is evident that the main features of the low-field spectrum between -11 and -15 ppm are conserved. However, even at 30°C , the -9.9 -ppm resonance is absent. A second feature of the spectrum in Figure 14 in contrast with that of unchanged

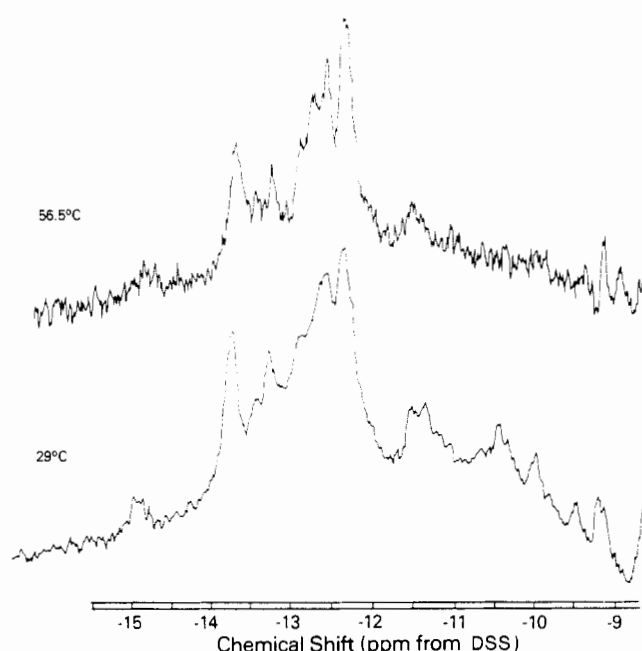


FIGURE 12: NMR spectrum of *E. coli* tRNA^{Phe} (pH 7.0) as a function of temperature. Solution conditions are the same as in Figure 11.

E. coli tRNA^{Val}, at similar pH and temperature, is the broadening of the entire spectrum. This broadening is observed also in the case of charged yeast tRNA^{Phe}, measured at 32°C , but no corresponding change in the -9.9 -ppm peak is evident compared to the uncharged molecule.

One explanation for such a general line broadening of the spectrum is a strong tendency toward aggregation of the aminoacylated species as opposed to the uncharged tRNAs due to the experimental conditions. It should be noted that previous study of aminoacylated vs. deacylated yeast tRNA^{Phe} in the -11 - to -15 -ppm region shows no significant broadening (Wong et al., 1973).

Discussion

We can summarize the basic observations of this investigation as follows. Concomitant with the absorbance and CD changes that occur when tRNA is exposed to acidic pH conditions (pH 5.5), we find in the case of three tRNA molecules a definite increase of one or two protons resonating at -9.9 ppm downfield from DSS. Since the majority of the resonances arising from ring protons in the secondary and tertiary structures of tRNA fall below -11 ppm, the -9 - to -11 -ppm spectral region represents a "window" between these protons and the much higher number corresponding to free amino groups and occur between -6 and -8 ppm (Wong et al., 1972; Patel and Canuel, 1976); this region should be useful for observing changes in tRNA and other RNAs as a function of the physical and chemical environment.

pH Behavior of -9.9 -ppm Resonance. The gain in intensity of the -9.9 -ppm resonance occurs at a pH that does not correspond to any normal base protonation. It is clear in the case of proteins that the pK s of side chains within a macromolecular structure can be considerably shifted from their normal values. A good example is the histidine ($pK_a = 7.1$) of human lysozyme (Cohen, 1969).

Similar shifts in the pK s of bases participating in hydrogen-bonded helical structures have been observed for some time (Steiner and Beers, 1961). Monomeric CMP has a pK for protonation of 4.2, and AMP has $pK = 3.6$, whereas the acid

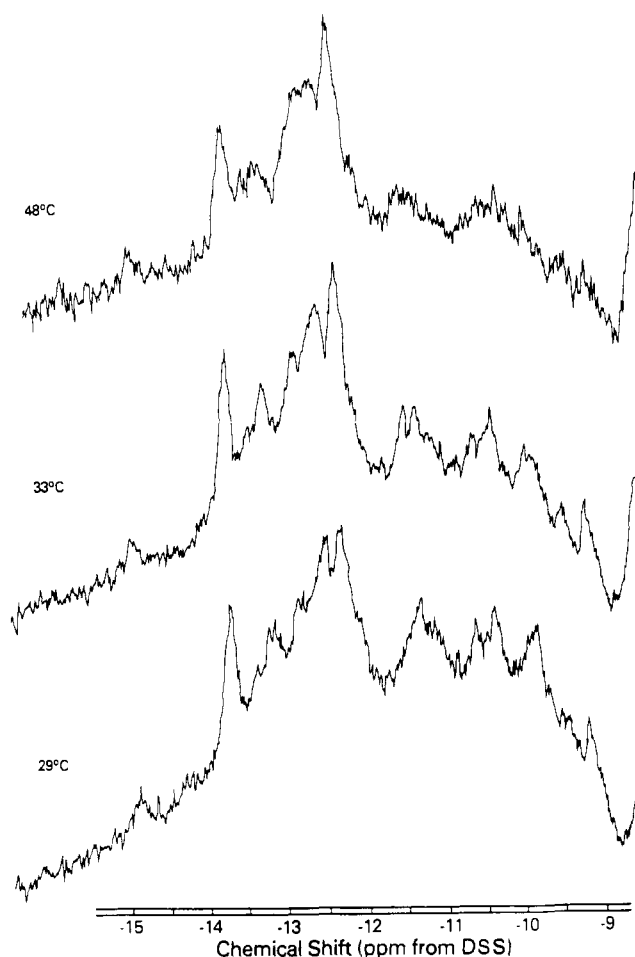


FIGURE 13: NMR spectrum of *E. coli* tRNA^{Phe} (pH 5.5) as a function of temperature. Solution conditions are the same as in Figure 10.

poly(C) helix has one pK at 5.7 and adenine in acid poly(A) a value near 6 depending on the salt concentration (Holcomb and Timasheff, 1968). Either C or A should represent the most likely site for protonation in tRNA near neutral pH, barring any unusual pK s associated with modified bases. The UV spectrum of A changes very little between pH 7 and 2, whereas protonation of C is accompanied by a substantial shift and change in intensity (P-L Laboratories Circular DR-10, 1973). Although it is difficult to draw conclusions about ΔA changes as small as those shown in Figure 7, it is worth noting that the shape of the $\Delta A(\lambda)$ profile corresponds fairly well to that expected for protonation of A but not C.

Assignment of the -9.9-ppm Resonance. Protonation of one or more nucleotide residues therefore results in the stabilization of additional hydrogen-bonded structures in three of the molecules tested. What is the nature of the change(s) occurring?

Two secondary structural ring protons have been assigned to the -9- to -11-ppm region: the N_1H of G and N_3H of U in G·U base pairs appear to resonate at -10.5 ppm downfield from DSS (Reid et al., 1975). Based on the approximate intrinsic positions of G(N_1H) and U(N_3H) in A·U and G·C Watson-Crick base pairs, -13.6 and -14.6 ppm, respectively (Kearns and Shulman, 1974; Patel and Tonelli, 1974; Kan et al., 1974), and the ring current shifts calculated from the known crystal structure of yeast tRNA^{Phe} (Sussman and Kim, 1976) no secondary structural base pairs contribute to the -9- to -11-ppm region.

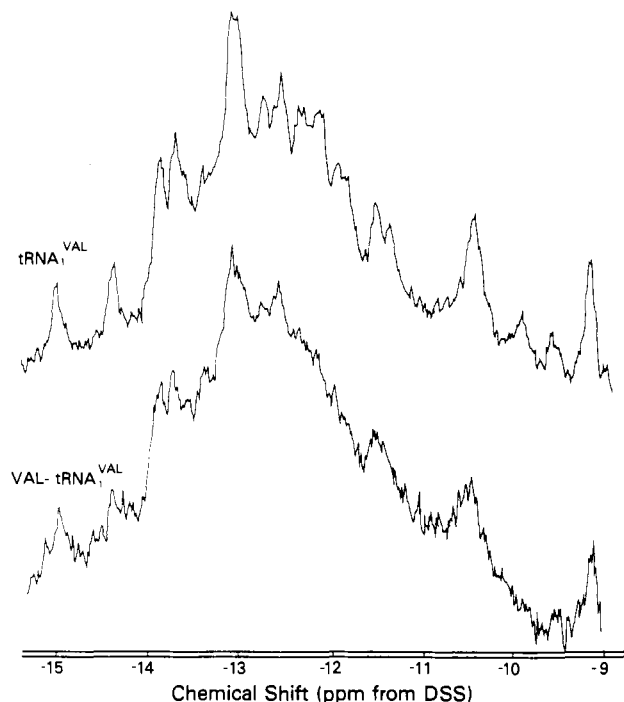
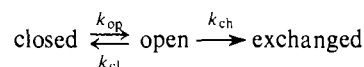


FIGURE 14: Comparison of the low-field proton NMR spectrum of *E. coli* tRNA^{Val} and valyl-tRNA^{Val}. Aminoacylation of *E. coli* valyl-tRNA^{Val} was conducted as described under Materials and Methods. Solution conditions: 1.5 mM tRNA, 100 mM NaCl, 10 mM MgCl₂, and 10 mM sodium cacodylate (pH 5.5), 30 °C.

We have found a model system involving non-Watson-Crick hydrogen-bonded protons, that exhibit resonances in this region. It is the acid oligo(A) double helix described here (Figure 5), in which a resonance at -9.4 ppm appears for chains containing six or more residues. The spectra are taken under conditions favoring formation of the acid (A) parallel stranded double helix (Eigen and Pörschke, 1970). In this instance, formation of the oligo(A) double helix depends upon the protonation of the N_1 position which results in stabilization of the non-Watson-Crick base-paired double helix.

Both the equilibrium and kinetic parameters of oligo(adenylic acids) have been determined by relaxation methods for various chain lengths by Pörschke and Eigen (1971). The exchange of base-paired protons in acid poly(A) has been investigated also by tritium exchange (Teitelbaum and Englander, 1975). According to Pörschke and Eigen (1971), the dissociation constant for double helical A(pA)₅ is $k_{op} \sim 1 \text{ s}^{-1}$ at 7 °C while the strand recombination rate k_R is about 10^6 L mol^{-1} at pH 4.2. Our NMR samples represent roughly 0.01 M in strands, so that the association rate (k_{cl}) is about 10^4 s^{-1} . Based on the standard exchange scheme for hydrogen-bonded protons (see Teitelbaum and Englander, 1975):



the closing rate is thus probably in excess of the chemical exchange, k_{ch} , since the exchange rate for the amino protons of A at pH 4.2 may be as slow as $1\text{--}10 \text{ s}^{-1}$ depending upon the base species present. This means that base-paired amino protons in acid oligo(A) helices exchange via a preequilibrium pathway, which for all-or-none dissociation implies that the ratio of the lifetimes of these protons in A(pA)₆ to that in A(pA)₅ is proportional to the stability constant for base pairing itself, S_{AA} , at the temperature of measurement. This factor

is complicated because of the contribution of base stacking to pairing, as discussed by Eigen and Pörschke (1970) and Appleby and Kallenbach (1973). However, a value $S_{AA} \sim 10$ at 5 °C is not unreasonable, and the detection of the exchanging resonance at -9.4 ppm for A(pA)₆ and not in A(pA)₅ can be accounted for on this basis. If the -9.4-ppm resonance corresponds to the N-H...N₇ hydrogen-bonded amino proton, the second amino proton paired to oxygen of phosphate (see Figure 5C) should lie 1–2 ppm upfield from this according to Katz and Penman (1966), or within the system of peaks centered near -8 ppm in Figure 8. Detailed transition studies of the proton NMR of these oligoadenylates will be reported elsewhere.

In terms of the ring current shielding present in the acid oligo(A) double helix (Rich et al., 1961), the N-H...N₇ proton would have an intrinsic value of -10.6 ppm, since the computed ring current effect amounts to roughly +1.2 ppm at the exocyclic amino NH position assumed to hydrogen bond with ring N₇ (see Figure 5C).

From the x-ray crystallographic studies of yeast tRNA^{Phe} (Kim et al., 1974; Robertus et al., 1974), two tertiary bonds consisting of the HNH...N type have been proposed. The A₉·A₂₃·U₁₂ triple is thought to represent two Watson-Crick base pairs between A₂₃·U₁₂ and two HNH...N base pairs between A₉ and A₂₃ (Figure 5A). The other tertiary bond of this type is G₁₅·C₄₈ which includes one HNH...O and one HNH...N hydrogen bond.

If we compute the ring current effect at the analogous site in the A₂₃·A₉ pair in yeast tRNA^{Phe} (+0.6 ppm) from the crystal-structure coordinates (Sussman and Kim, 1976), this proton would be expected to appear in the vicinity of -10 ppm in yeast tRNA^{Phe}. These calculated values are predicted from approximate theoretical values (Giessner-Prettre et al., 1976) in the first place, and assume a tRNA structure in solution identical with that in the crystals. Nevertheless, the thrust of the available evidence is such as to implicate the protonation of ring N₁ of A₉ in stabilizing residues A₂₃ and A₉ in an acid (A)-like base pair at acidic pH near 6.

If this interpretation, which is somewhat tentative at present, is correct, the behavior of *E. coli* tRNA^{Phe} and yeast tRNA^{Phe} is easy to understand. At pH 7, in the absence of the protonation of A₉ at N₁ of the ring, the segment of acid (A) structure cannot form because of the requirement for the positive charge on the ring to stabilize the double helix of AH⁺·AH⁺ by electrostatic interaction with the vicinal phosphates (see Rich et al., 1961; Holcomb and Timasheff, 1968). Somewhat above pH 6, A₉ protonates (A₂₃ cannot, because it participates in the U₁₂·A₂₃ Watson-Crick pair), and two added amino protons in the low-field spectrum appear at -9.9 ppm.

While this scheme satisfactorily accounts for the intensity change of two in yeast tRNA^{Phe} and possibly in *E. coli* tRNA^{Phe} as well, it does not explain the behavior of *E. coli* tRNA^{Val} which appears to increase by one proton at -9.9 ppm. However, the added resonance in *E. coli* tRNA^{Val} is exceptionally labile to begin with, and the detailed structure may not conform precisely to the yeast tRNA^{Phe} crystal structure in any event. What is more significant is that three tRNA molecules with highly similar tertiary interactions differ in the stability of that structure, and that these differences can be analyzed by proton NMR spectra in H₂O in the -9- to -11-ppm region.

Acknowledgment

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