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^{1}H Nuclear Magnetic Resonance Studies of Transfer RNA: The Methyl and Methylene Resonances of Baker's Yeast Phenylalanine Transfer RNA and Its Fragments†

Lou S. Kan, Paul O. P. Ts'o,* M. Sprinzl, F. v. d. Haar, and F. Cramer

ABSTRACT: The methyl and methylene resonances of intact Baker's yeast $tRNA^{Phe}$ and its four key fragments in D_2O solution with Mg^{2+} have been measured by both 220- and 360-MHz spectrometers in a temperature range of from 16 to 98.5 °C. Totally, 12 methyl and 2 methylene resonances in $tRNA^{Phe}$ can be unambiguously assigned at high temperatures. Therefore, the profile of chemical shifts vs. temperature of each resonance can be measured. Four conclusions can be reached by this study based on chemical shift and line width: (i) the anticodon loop protrudes from the molecule and does not associate with any parts of the molecule; (ii) the presence of Mg^{2+} greatly stabilizes the native form, not only because the T_m is higher than that without Mg^{2+} (Kan, L. S., et al. (1974), *Biochem. Biophys. Res. Commun. 59,* 22) but also because the transition profile is more narrow; (iii) the residues D's, m^2_2G, T, m^1A, and perhaps m^7G, are involved in the tertiary structures of native tRNA; (iv) the T residue may have two different conformations, probably with respect to the mode of stacking to G_{53} in the native state. Based on the refined atomic coordinates of $tRNA^{Phe}$ in orthorhombic crystal and on the recent advances in the distance dependence of the ring-current magnetic field effects (Giessner-Prettre, C., et al.(1976), *Biopolymers 15,* 2277), a computed shielding effect ($\Delta\delta$) for these high field resonances was made. The computed $\Delta\delta$'s were compared with the observed $\Delta\delta$'s and the comparison indicates that the conformation of yeast $tRNA^{Phe}$ in aqueous solution is closely similar but not identical with that found in the crystal, especially in the TψC and D regions.

Recently, the structure of yeast phenylalanine transfer ribonucleic acid ($tRNA^{Phe}$) in crystalline state has been clearly elucidated by x-ray diffraction studies (Kim et al., 1974; Ladner et al., 1975a). Furthermore, the three-dimensional coordinates of all atoms (except hydrogen) in this tRNA molecule have been reported by several laboratories (Quigley

† From the Division of Biophysics, Johns Hopkins University, Baltimore, Maryland 21205, and Abteilung Chemie, Max-Planck-Instituts fur Experimentalle Medizin, 34 Gottingen, Germany. *Received January 3, 1977.* This is the second paper in a series. The first paper in this series was: Kan, L. S., et al. (1975), *Biochemistry 14,* 3278. We thank the National Institutes of Health (GM016066-06,07), National Science Foundation (GB-30725X), and NATO (No. 701) for financial support for this work.

et al., 1975; Ladner et al., 1975b; Sussman and Kim, 1976; Stout et al., 1976; see review by Rich and RajBhandary, 1976). The $tRNA^{Phe}$ structures determined from these two crystal forms are very similar to each other. Therefore, a very crucial question arises: does the common conformation of $tRNA^{Phe}$ determined in crystalline state also exist in aqueous solution? At present, information obtained from nuclear magnetic resonance (NMR)[1] studies can provide a direct and defined answer to this question.

Two spectral regions in the ^1H NMR spectrum of $tRNA^{Phe}$ can be investigated for quantitative conformational information. The first region contains the very low field NH-N hydrogen-bonded proton resonances in H_2O. Our study of yeast $tRNA^{Phe}$ in this region will be reported in a subsequent paper (Kan and Ts'o, 1977). The second region is described in this communication, in which the high-field (1-4 ppm) resonances of the methyl/methylene protons from the minor nucleosides in yeast $tRNA^{Phe}$ in D_2O are investigated. The emphasis of this study is not on base pair region which can be studied by the NH-N hydrogen-bonded proton resonances but rather on the loop regions and tertiary structure of tRNA molecule (Kan et al., 1974; Kastrup and Schmidt, 1975; Reid and Robillard, 1975; Daniel and Cohn, 1975, 1976), especially the D, anticodon, and TψC loops on $tRNA^{Phe}$.

In addition to the intact $tRNA^{Phe}$ molecule, four important fragments have also been investigated. These fragments are not only very useful in the assignment of the methyl/methylene resonances of the intact $tRNA^{Phe}$, but also provide information on the conformation of the whole molecule of $tRNA^{Phe}$.

Finally, by taking advantage of recent developments in the distance dependence of the ring-current magnetic effect of the bases (Griessner-Prettre et al., 1976) and in the refined atomic coordinates of yeast $tRNA^{Phe}$ in the crystalline state (A. Rich, private communication), a quantitative comparison between the calculated shielding effects ($\Delta\delta$) and the observed shielding effects was made. This study suggests that the conformation of yeast $tRNA^{Phe}$ in aqueous solution is grossly, but not totally, identical with that determined in the crystalline state, especially in the TψC and D regions.

Materials and Methods

Chemicals. Highly purified $tRNA^{Phe}$ was obtained from Baker's yeast by a previously published procedure (Schneider et al., 1972). Four fragments, 1-45 (5′ ¾ $tRNA^{Phe}$), 47-76 (3′ ¼ $tRNA^{Phe}$), 31-42 (anticodon loop), and 54-57 (T-ψ-C-G) as well as mononucleotide Y were obtained by partial enzymic digestion of $tRNA^{Phe}$. The digestion reaction, purification, and identification of these compounds were published elsewhere (Sprinzl et al., 1976). The 2′-*O*-methylguanosine (Gm) (and 2′-*O*-methylguanosine 3′-monophosphate, Gmp) and 2′-*O*-methylcytidine (Cm) were gifts from Drs. P. S. Miller and D. W. Cochran in our laboratory, respectively. Other modified mononucleosides (or -nucleotides) of $tRNA^{Phe}$, namely, N^2-methylguanosine (m^2G), dihydrouridine (D), $N^{2,2}$-dimethylguanosine ($m^2{}_2G$), 5-methylcytidine (m^5C), 7-methylguanosine (m^7G), ribothymidine (T), and 1-methyladenosine (m^1A), were purchased from either Sigma Chemical Co., St. Louis, Mo., or from Terra-Marine Bio-Research, La Jolla, Calif. These mononucleosides (or -nucleotides) were used for ^1H NMR investigation without any further purification. $MgCl_2 \cdot 6H_2O$ (ACS certified grade) was obtained from Fisher Scientific Co., Fair Lawn, N.J., was baked at 110 °C overnight, and then recrystallized in D_2O. D_2O, 99.83%, was purchased from Bio-Rad Laboratories, Richmond, Calif.

^1H NMR Spectroscopy. The ^1H NMR spectra were recorded on a Varian HR 220-MHz ^1H NMR spectrometer located at the University of Pennsylvania, Philadelphia, Pa., and a Bruker 360-MHz ^1H NMR spectrometer at Stanford University, Stanford, Calif., both equipped with a variable-temperature accessory and fast Fourier transform unit. A trace amount of *tert*-butyl alcohol was added to the sample as an internal standard, but all reported values of the chemical shifts have been converted to the DSS standard (Borer et al., 1975). The chemical shift data of well-separated signals are accurate within 0.01 ppm. The negative signs of all chemical shifts are omitted.

All ^1H NMR samples of these mononucleosides (or -nucleotides), intact $tRNA^{Phe}$, and its fragments were made in D_2O with 0.01 M potassium phosphate buffer, pH 6.5-7.0, unless specified otherwise. The concentration of mononucleosides (or -nucleotides) was 0.001 M, except m^2G and $m^2{}_2G$, which are very insoluble in water. Therefore, their saturated solutions were investigated. No $MgCl_2$ was added to the mononucleoside (or -nucleotide) solutions. The concentrations of the intact $tRNA^{Phe}$ fragments are 36 A_{260} units/0.35 mL for fragment 1-45, 33.6 A_{260} units/0.35 mL for fragment 45-76, 30 A_{260} units/0.4 mL for fragment 54-57, and 90 A_{260} units/0.4 mL for fragment 31-42 and have been reported previously (Kan et al., 1975). Two intact $tRNA^{Phe}$ samples were prepared for this study: 70 A_{260} units/0.4 mL (~0.35 mM) for 220-MHz and 80 A_{260} units/0.3 mL (~0.5 mM) for 360-MHz ^1H NMR spectrometer. All NMR samples of intact tRNA and its fragments contain 0.01 M $MgCl_2$, unless otherwise specified.

The amino acid acceptor activity of $tRNA^{Phe}$ molecules was reexamined after the temperature studies of $tRNA^{Phe}$ by the method of Sprinzl and Cramer (1973). The acceptor activity of yeast $tRNA^{Phe}$ was rapidly reduced when temperatures were above 86 °C. Since the activity of $tRNA^{Phe}$ cannot be retained after heating at high temperature in the ^1H NMR probe, the thermal experiments must be done by proceeding from low to high temperatures, and it is not recommended that the heated sample be reused.

The Ring-Current Effect Calculation. The calculations of the ring-current effect on the methyl/methylene protons from their surrounding bases in the yeast $tRNA^{Phe}$ are based on (i) the tRNA structure in crystal as determined by x-ray diffraction studies and (ii) the profiles of the shielding/deshielding effects (in ppm) vs. the vertical (z) and radial distance (ρ) from a given base plane nearby. The z and ρ were defined as shown in Figure 1; if A is the proton of interest, z is the vertical distance from A to a base plane (i.e., AB, B is on the base plane); and ρ (or ρ') is equal to BO (or BO′) when O (or O′) is the center of a six-membered (or five-membered) ring. These profiles have been recently computed with z values from 0 to 8 Å and with ρ values from 0 to 10 Å (Giessner-Prettre et al., 1976). The atomic coordinates of yeast $tRNA^{Phe}$ in orthorhombic crystal are kindly provided by Dr. A. Rich of MIT. Since the resonances of both methyl and methylene groups on the modified bases show simple patterns in the ^1H NMR spectra, it is safe to assume that these protons in these groups are in magnetically equivalent environment. Therefore, the ring-current effect is calculated for the "average" position of methyl/methylene protons within the group. For methyl protons, the average position is the projection point of any proton to the bond line of $C\text{-}CH_3$ (or $N\text{-}CH_3$, or $O\text{-}CH_3$) with

[1] Abbreviations used: NMR, nuclear magnetic resonance; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; UV, ultraviolet.

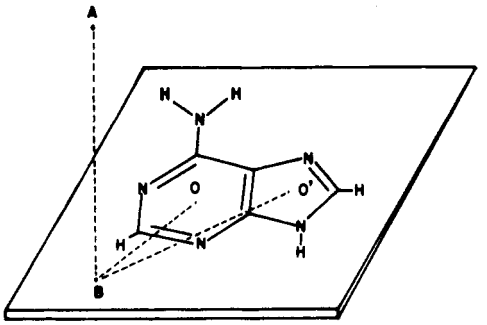

FIGURE 1: A diagram of the geometrical relationship between a given proton (A) and a nucleic acid base (i.e., adenine). The distances AB and BO/BO′ derived from the coordinates of the tRNA structure provide the needed parameters for the computation of the ring current magnetic field effects (Giessner-Prettre et al., 1976).

the average C–H bond length adopted as 1.09 Å and with an average bond angle of C–C–H adopted as 109° 30′. Therefore the distance from the projection point to methyl carbon atom will be equal to 1.09 Å × cos (180–109° 30′), or 0.364 Å on the line of *C*-CH_3 (or *N*-CH_3, or *O*-CH_3) bond. The average position of two methylene protons is assumed to be the middle point between these two protons. Therefore, the distance from the average position to methylene carbon atom will be equal to 1.09 Å × cos (109° 30′/2), or 0.629 Å. All of these calculations were done using a DEC-10 computer at the Johns Hopkins University Computing Center. The details of these calculations, as well as computer programs, will be published elsewhere (Kan, Ts'o, Ts'o, Giessner-Prettre, and Pullman, manuscript in preparation).

Results

The Assignment of the Methyl and Methylene Resonances in the Intact tRNA^{Phe} and Its Fragments at High Temperature. At high temperatures, the chemical shift values of the residues in an oligo(poly)nucleotide are close to those of their corresponding mononucleosides due to the loss of the intra- and intermolecular interactions. Therefore, the first step for assignment of these high-field resonances from tRNA^{Phe} and its fragments is to compare their spectra with those from monomers at high temperature. All modified mononucleosides (-nucleotides) found in tRNA^{Phe} molecules (Figure 2) were investigated separately at high temperatures, and their chemical shift data of methyl or methylene proton resonances are marked on the top row in Figure 3. This knowledge from the mononucleosides (-nucleotides) together with the sequence data provides the basis for the unambiguous assignment of the methyl, methylene resonances in tRNA^{Phe} fragments.

For instance, fragment 54–57 has only one high-field resonance which has a chemical-shift value similar to the methyl resonance of monomer T (second row, Figure 3). Undoubtedly, this peak can be assigned to the T residue in fragment 54–57, the only residue possessing a methyl group in this fragment. Fragment 47–76 contains three methyl modified bases; m^1A (near 3.9 ppm) was obscured by the impurities, and only two methyl resonances can be seen (Figure 3). One can readily assign the downfield signal to m^5C_{49} and the upfield one to T, based on the argument that the methyl resonance of T in the fragment should not be located at a lower field position than that of the T monomer. The fourth row in Figure 3 shows all methyl resonances from Cm, Gm, Y (which contains three signals designated as Y, Y′, and Y″) and m^5C in fragment 31–42. Assignment of these resonances can be made by comparison with the monomers and the smaller fragments from

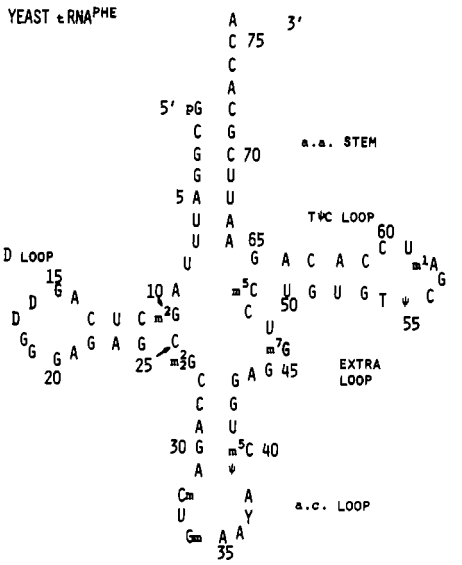

FIGURE 2: The cloverleaf structure of Baker's yeast tRNA^{Phe}.

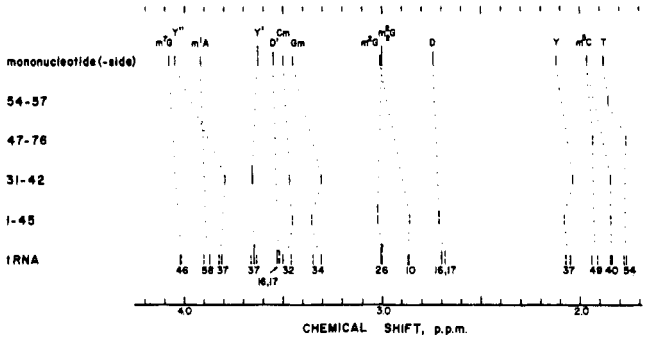

FIGURE 3: The schematic plot of the assignment of methyl/methylene resonance signals in the intact Baker's yeast tRNA^{Phe} with/without 0.01 M Mg^{2+} at high temperature (98.5/79 °C). The solid sticks represent ^{1}H NMR signals which are taken from a sample with 0.01 M phosphate buffer (pH 7) and the broken sticks represent the ^{1}H NMR signals from the same buffer solution with additional 0.01 M $MgCl_2$. The relative heights of these sticks reflect the number of protons of each peak; namely, the tallest stick represents six protons, medium height represents four, and the shortest stick represents three protons. The methyl/methylene ^{1}H NMR resonances of mononucleotides (-sides) are shown in the first row. Most of them contribute a singlet from the methyl group and the names of the modified bases are given above the resonances. But the Y base shows four (sometimes three) methyl singlets which are symbolized by Y = C_{11}–Me, Y′ = NCOOMe and CCOOMe, and Y″ = N_3–Me. Only the D base contains two methylene triplets, namely, D = C_5–methylene and D′ = C_6–methylene. The spectra of this monomers have been taken within a temperature range of 70–80 °C. The last row shows the ^{1}H NMR signals of these modified bases in intact tRNA^{Phe} (with/without Mg^{2+}). These assigned signals (see text) are identified by the nucleotide sequential numbers directly below each peak. There are four tRNA^{Phe} fragments, identified by nucleotide sequential numbers for the first and last nucleotides, listed between the first and last rows. The temperatures at which the spectra were taken are the following: 70 °C for fragment 54–57, 90 °C for fragment 47–76, 63 °C for fragment 31–42, and 93 °C for fragment 1–45. Measurements were made in sufficiently high temperature to abolish all the secondary structures of these fragments. Usually, larger the fragments, higher the temperature needed to reach this condition.

this dodecanucleotide, including the dodecanucleotide without the Y base (excision by mild acid treatment). Such a comparative study and assignment has been made previously (Kan et al., 1975). Fragment 1–45, the 5′ ¾ molecule (Figure 2), contains m^2G, two D's, $m^2{}_2G$, Cm, Gm, Y, and m^5C bases. The signals from Cm, Gm, Y, and m^5C can be readily assigned since they are included in the fragment 31–42.The D base contains two triplets, designated as D′ and D; in the spectrum

TABLE I: The Chemical Shifts of the Methyl and Methylene Resonances from Intact Yeast $tRNA^{Phe}$ (0.35–0.5 mM, 0.01 M $MgCl_2$, 0.01 M Potassium Phosphate Buffer, pH 6.5–7.0).

Minor bases	Chemical shifts (δ, ppm)[a] tRNA Denatured (85–98.5 °C)	Native (~50 °C)	Monomers[f]	$\Delta\delta$[b]
m^2G_{10}	2.86	2.74	3.11	0.37
$D_{16,17}$ (C_5)	2.68	2.47	2.74	0.27
(C_6	3.52	3.03[d]	3.56	0.53
$m^2{}_2G_{26}$	3.00	2.64[e]	3.10	0.46
Cm_{32}	3.46	3.58[e]	3.50	−0.08
Gm_{34}	3.35	3.57	3.44	−0.13
Y_{37} (CCH_3)	2.07	2.08	2.16	0.08
(NCH_3)	3.87	3.90	4.07	0.17
($COOCH_3$'s)	3.63	3.66	3.64	−0.02
	3.66	3.68	3.67	−0.01
m^5C_{40}	1.85	1.56	1.95	0.39
m^7G_{46}	4.00[c]		4.09	
m^5C_{49}	1.91	1.58	1.95	0.37
T_{54}	1.78	1.49 and 1.01	1.88	0.87 and 0.39
m^1A_{58}	3.86	3.78	3.93	0.15

[a] Negative sign omitted. [b] $\Delta\delta = \delta_{tRNA,\ native\ state} - \delta_{monomer}$. [c] These data were obtained at 85 °C, 360-MHz NMR spectrometer; all the rest were from 220-MHz NMR spectrometer, 98.5 °C. [d] Tentative assignment. [e] These data were at 68 °C, all others were 50 °C. [f] These monomer data were taken between 63 to 80 °C.

of this fragment 1–45, the signals of D′, Y″, and Y′ are masked by the impurity. The assignment of the D resonance does not depend only on the chemical shift but also on the consideration that this resonance is a triplet. However, no distinction can be made between base D_{16} and base D_{17} at present. The remaining two singlets, $m^2{}_2G$ and m^2G, can be assigned unambiguously due to the difference in intensity; the $m^2{}_2G$ having two identical methyl groups is twice as intense as the m^2G having only one methyl group (fifth row, Figure 3).

The bottom row in Figure 3 contains the high-field resonances of intact $tRNA^{Phe}$ with (broken stick) and without (solid stick) Mg^{2+} at high temperature. The chemical shifts of most of the signals are very close to their counterparts in the fragments, allowing a straightforward identification. It should be noted that, by comparing fragment 47–76 (which contains m^5C_{49}), fragment 31–42 and fragment 1–45 (both contain m^5C_{40}), the chemical shifts from m^5C_{40} and m^5C_{49} can be differentiated. The resonances from m^7G, m^1A, and D′ (a triplet) were either absent from the fragments or obscured by the impurities in the samples; their resonances in the intact $tRNA^{Phe}$ can be assigned by a direct comparison with the monomers. This comparison is facilitated by the fact that D′ is a triplet and the resonances of m^7G and m^1A are located at very low field positions, ~4.0 and ~3.8 ppm, respectively (Figure 3). Thus, all the methyl and methylene resonances of $tRNA^{Phe}$ and its fragment can be assigned at high temperature with confidence. The chemical shifts of all the assigned methyl and methylene resonances of yeast $tRNA^{Phe}$ are tabulated in Table I.

Studies of the Methyl and Methylene Resonances from the Fragments of $tRNA^{Phe}$. (a) Fragment 54–57 and Fragment 31–42. The tetranucleotide, T-ψ-C-G(54–57), exhibits only one methyl resonance (T), which shows no change either in chemical shift or line width within a temperature range of 5 to 80 °C (Figure 4d). The data indicate that the methyl group of the T in the tetramer has a similar chemical shift in the monomer (within 0.05 ppm) and is much less shielded than the T in the fragment 47–76 (0.2–0.8 ppm) and in the intact $tRNA^{Phe}$ (0.3 to 0.8 ppm).

The dodecanucleotide, fragment 31–42, has been previously investigated extensively in the absence of Mg^{2+} (Kan et al., 1974, 1975). In Figure 5, all of the chemical shifts of the methyl resonances in this fragment are compared with those from the monomer and the intact $tRNA^{Phe}$ in the presence or the absence of Mg^{2+}. The chemical shift of the 2′-*O*-methyl group of Cm_{32} monomer is about the same as those from the fragment and the intact $tRNA^{Phe}$ at denaturing temperature (above 40 °C for the fragment 31–42 and $tRNA^{Phe}$ without Mg^{2+}; above 80 °C for the $tRNA^{Phe}$ with Mg^{2+}). Below this denaturing temperature, the 2′-*O*-methyl group of Cm_{32} in the fragment and in the tRNA tends to be deshielded by 0.1–0.15 ppm. This downfield shifting phenomenon has been previously observed and explained in the study of 2′-*O*-methyl-poly(A) (Alderfer et al., 1974) because the shielding of the 2′-*O*-methyl group comes from the anisotropy of the neighboring bases. Upon the stacking of the neighboring bases at low temperature, the shielding of the 2′-*O*-methyl group located at the furanose is thus reduced. Such a phenomenon is even more pronounced for the 2′-*O*-methyl group of the Gm_{34} (Figure 5b). At a sufficiently high temperature (60–80 °C, depending on the presence or the absence of Mg^{2+}), the 2′-*O*-methyl group of Gm_{34} in the fragment 31–42 and in the $tRNA^{Phe}$ is slightly more shielded (~0.1 ppm) than that of the monomer; but at low temperature, the 2′-*O*-methyl group in the fragment and $tRNA^{Phe}$ is more deshielded. Such observation was also made in the hexamer, Gm-A-A-Y-A-ψp (Kan et al., 1975).

The three methyl resonances from the Y base have been previously assigned (Kan et al., 1975) as follows: Y, C-CH_3; Y′, N-$COOCH_3$ and C-$COOCH_3$; Y″, N-CH_3. The methyl resonances of the C-CH_3, N-$COOCH_3$, and C-$COOCH_3$ in the monomer, the fragment, and the $tRNA^{Phe}$ (with or without Mg^{2+}) are shielded to about the same extent and are independent of temperature (Figure 5c–e). The methyl resonance of the N-CH_3 is also temperature independent, but is shielded more in the fragment and in the $tRNA^{Phe}$ than in the monomer Y base (Figure 5c). This situation has been carefully evaluated (Kan et al., 1974, 1975). This is an indication of the geometry of the stacking of the base in the fragment and in the $tRNA^{Phe}$

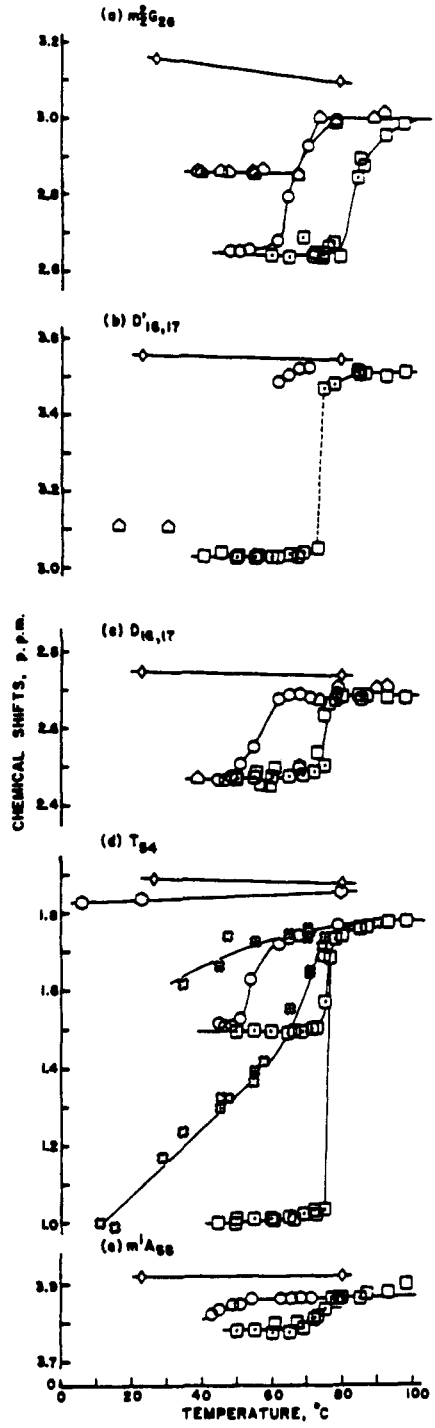

FIGURE 4: The chemical-shift data of the methyl proton resonances from (a) m^2_2G, (b) D′, (c) D, (d) T, and (e) m^1A in the monomer, the intact $tRNA^{Phe}$, and its fragments vs. temperature. The explanation of the symbols used is as follows: (◇) the monomer; (○) fragment 54–58; (△) fragment 1–45 in 0.01 M $MgCl_2$; (▫) fragment 47–76 in 0.01 M $MgCl_2$; (○) yeast $tRNA^{Phe}$, (□) yeast $tRNA^{Phe}$ in 0.01 M $MgCl_2$. ⊕, ⊙, and ▣ mean that the data were obtained by a 360-MHz spectrometer.

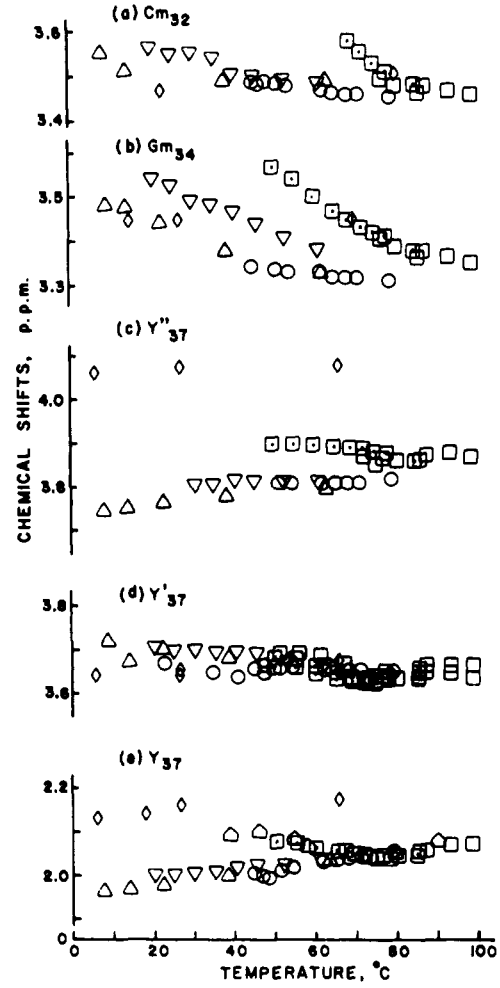

FIGURE 5: The chemical-shift data of the methyl proton resonances from (a) Cm, (b) Gm, (c) Y″, (d) Y′, and (e) Y, of the monomer, intact $tRNA^{Phe}$ and fragment 31–42 and fragment 1–45 vs. temperatures, where △ represents fragment 31–42 and ▽ represents the fragment 31–41 in 0.1 M NaCl and 0.1 M $MgCl_2$. The remaining symbols have the same meaning as in Figure 4.

as well as an indication of a high degree of freedom of the Y base in the intact $tRNA^{Phe}$.

(b) Fragment 47–76. Owing to the impurity acquired in the preparation of the fragment (three multiplets at 3.40–4.50 ppm), only the methyl resonance of m^5C and T can be studied in this quarter molecule of $tRNA^{phe}$ (Figure 6). A few selected 220-MHz spectra of fragment 47–76 are shown in Figure 6b from 48 to 79 °C. At 79 °C, both m^5C and T resonances are narrow in their line width; at 74 °C, the T signal begins to broaden; at 71 °C, the T signal is split into two peaks, located at 1.65 and 1.76 ppm.

The high-field resonance (1.65 ppm at 71 °C) continues to shift upfield upon lowering the temperature, while the low-field resonance (1.71 ppm at 71 °C) shifts considerably less. Thus, these two peaks are farther apart from each other at reduced temperatures (Figure 6b). At 48 °C, the line width of the high-field signal (at 1.35–1.40 ppm) appears to be much narrower than the low-field signal (~1.7 ppm), though at 70 °C these two signals have similar line widths. Investigation of this fragment at 360 MHz frequency confirmed the observation about T measured at 220 MHz (Figure 6a). At this frequency, the T resonance is also quite broad at 75 °C, and two peaks begin resolvability at 70 °C. At 65°, the two signals become clearly evident; and at 55 °C the high-field signal (~1.40 ppm) remains relatively narrow in line width, while the low-field signal (centered at 1.73 ppm) becomes very broad. When the temperature was lowered continuously, the highfield peak of T methyl resonance kept on moving upfield, finally reaching 1.00 ppm at temperature below 20 °C (Figure 4d), while the low-field peak also moved upfield to ~1.6 ppm but broadened to become unrecognizable below 38 °C.

The methyl resonance of m^5C_{49} in fragment 47–76 is not

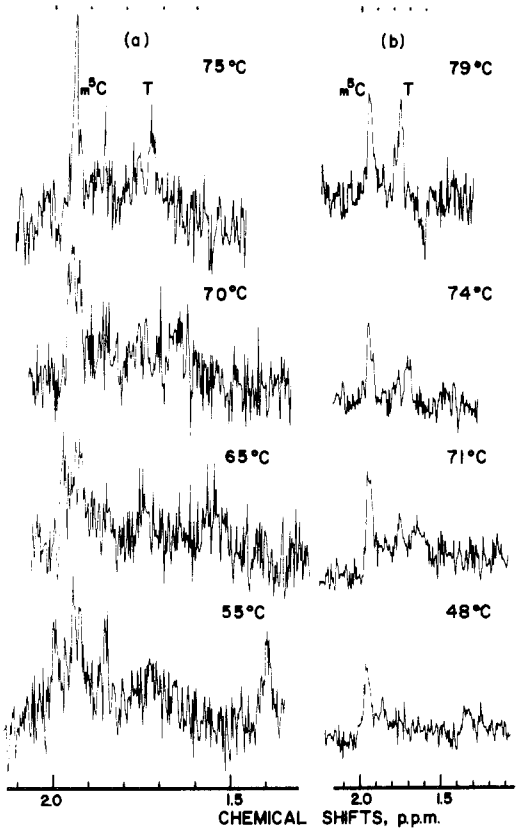

FIGURE 6: (a) The 360-MHz (with acquisition time 1 s, 256–512 transients) and (b) 220-MHz (with acquisition time 0.8 s, 10 blocks (100 transient in one block) were accumulated) spectra of the m^5C_{49} and T methyl proton resonances of fragment 47–76 at different temperatures.

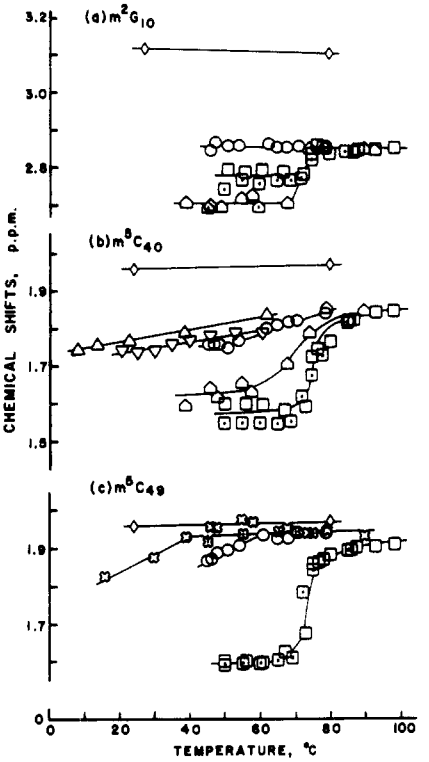

FIGURE 7: The chemical-shift data of methyl proton resonances from (a) m^2G, (b) m^5C_{40}, and (c) m^5C_{49} of the monomer, intact $tRNA^{Phe}$ and its fragments vs. temperatures. All of the symbols have the same meaning as in Figure 4.

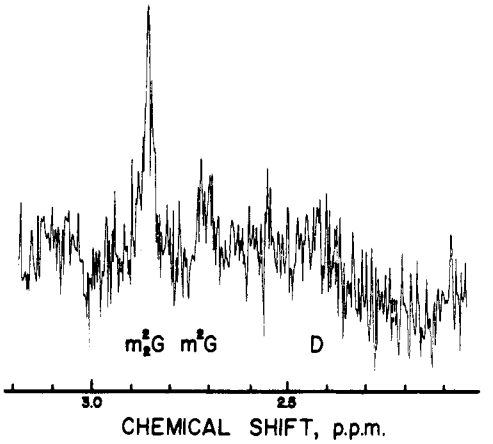

FIGURE 8: A partial ^1H NMR spectrum (only m^2_2G, m^2G, and D are shown in the range of 2 to 3.2 ppm) of fragments 1–45 at 40 °C. This spectrum was taken at 360 MHz with acquisition time 1 s, 512 transients.

shifted until the temperature is lowered about 40 °C. At that temperature, it begins to shift upfield (Figure 7c) accompanied with extensive line width broadening observed at 220-MHz frequency. The line width of the m^5C_{49} signal remains narrow from 79 to 48 °C when measured at 220 MHz or from 75 to 55 °C measured at 360 MHz (Figure 6).

(c) Fragment 1–45. The impurities in this preparation of fragment 1–45, the ¾ molecule, also obstructed the signals of Y′, Y″, and D′ at low field and only permitted the observation on m^2G, D, m^2_2G, C-CH_3 of Y base, and m^5C_{40} as well as Cm_{32} and Gm_{34} at high temperature. From 16 to 93 °C, the chemical shifts and the line widths of m^2G_{10}, D (from both D_{16} and D_{17}), C-CH_3 of Y base and m^5C_{40} from this half molecule are nearly the same as those from the intact $tRNA^{Phe}$. On the other hand, the signal of m^2_2G in the fragment 1–45 is different from that in the intact $tRNA^{Phe}$ (Figure 8). Even at a low temperature of 40 °C, the methyl resonance of m^2_2G in the fragment does not become broadened and maintains at 2.86 ppm (Figure 4a) while the signal of m^2_2G in the $tRNA^{Phe}$ becomes broadened below 65–70 °C (with Mg^{2+}) (Figures 9 and 10) or 50 °C (without Mg^{2+}) (Kan et al., 1974) and levels off at a higher field position of 2.60 ppm.

Below 30 °C, the region of 3.11 ppm of the fragment 1–45 spectrum appears to contain a signal which can be from the D′ group. The reason for this suggestion is that the tentatively assigned D′ signal from the $tRNA^{Phe}$ is located at 3.03 ppm (Figures 9 and 10). This signal at 3.11 ppm persists at the same position as low as 16 °C, though, at that temperature, all other signals become too broad for any useful study.

Studies of the Methyl and Methylene Resonances from the Intact $tRNA^{Phe}$. A few selected 220-MHz ^1H NMR spectra of the intact $tRNA^{Phe}$ with 0.01 M $MgCl_2$ from 40 to 98.5 °C are shown in Figure 9. The identity of every signal at 98.5 °C is marked on the top of the peak and is extended to the signals measured at lower temperatures by the dotted lines. At 98.5 °C (Figure 9a), the spectral patterns and chemical shifts of $tRNA^{Phe}$ with Mg^{2+} are very similar to that of $tRNA^{Phe}$ without Mg^{2+} at 79 °C (Kan et al., 1974). This temperature differential of 20 °C in reaching the same state of denaturation is due to presumably the presence of Mg^{2+}, which stabilizes the native form of $tRNA^{Phe}$. At 80 °C (Figure 9b), while the $tRNA^{Phe}$ in Mg^{2+} is still in the denatured state shown by the transition profile in Figures 4 and 7, the signals from m^2_2G and m^2G begin to shift upfield, and all signals begin to broaden, except those from the Y base. At 73 °C, $tRNA^{Phe}$ is at the

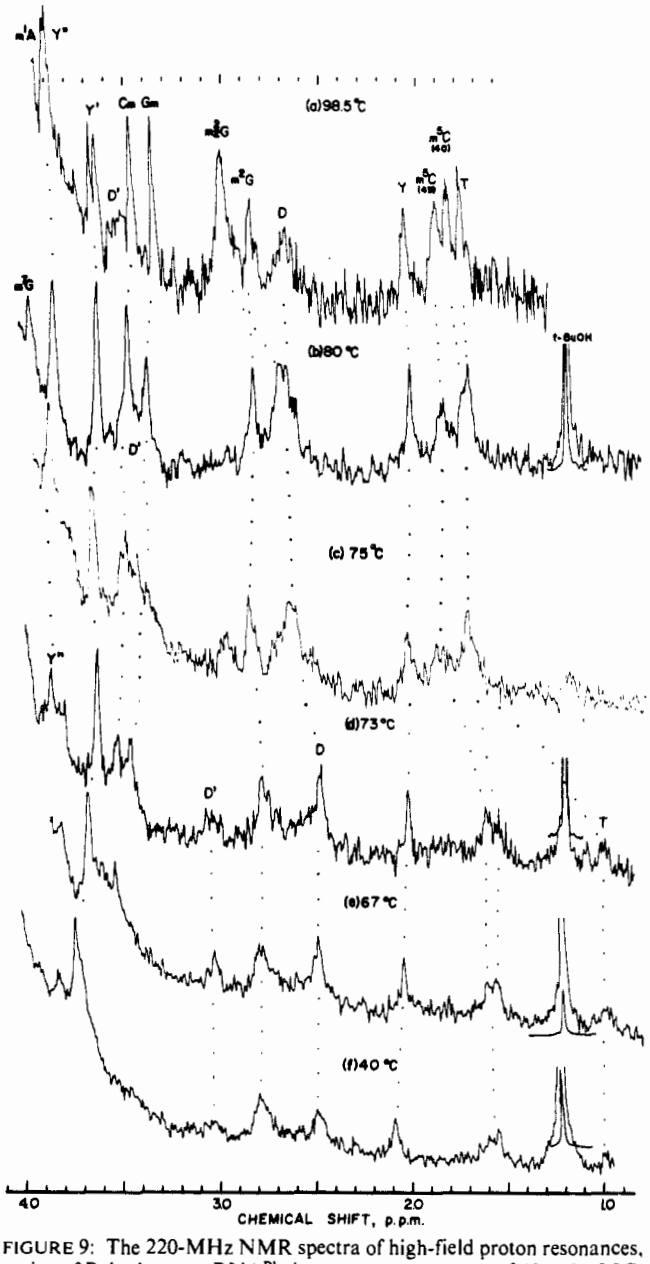

FIGURE 9: The 220-MHz NMR spectra of high-field proton resonances, region of Baker's yeast tRNA^{Phe} in a temperature range of 40 to 98.5 °C. The experimental conditions are: acquisition time 0.8 s, 8 blocks (100 transients in one block) for spectrum a, 5 blocks (200 transients in one block) for spectra b and c, 6 blocks (200 transients/block) for spectrum d, and 10 blocks (200 transient/block) for spectra e and f.

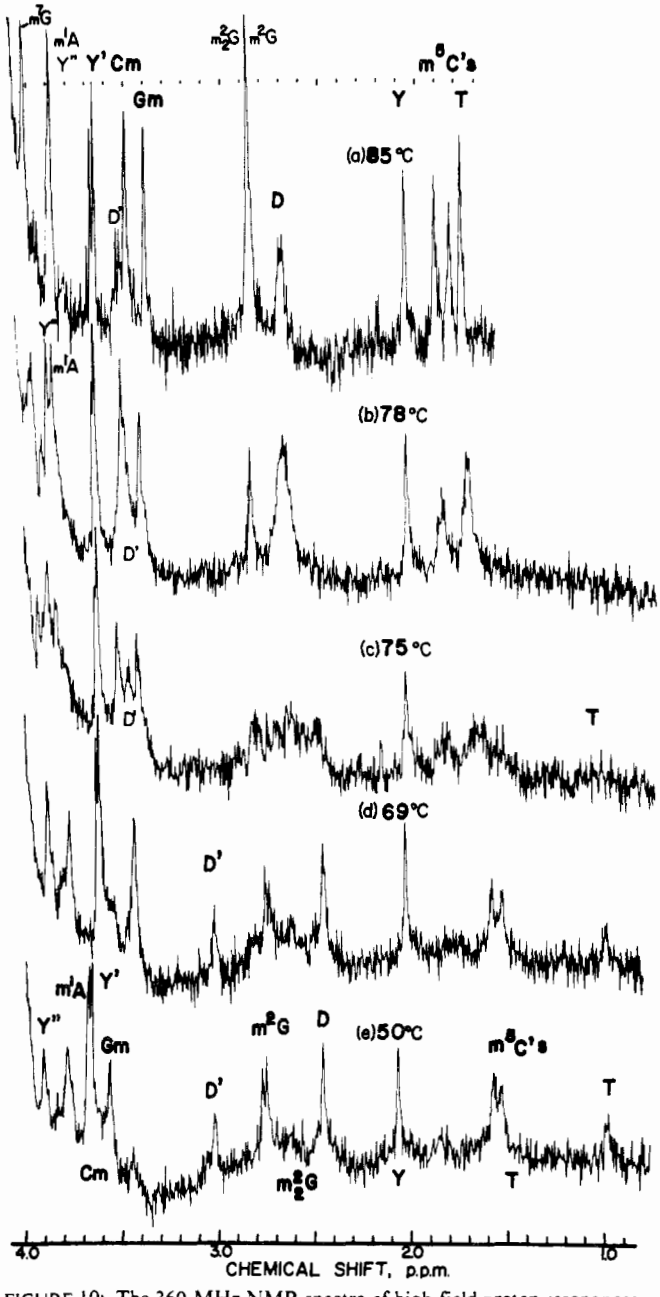

FIGURE 10: The 360-MHz NMR spectra of high-field proton resonances, region of Baker's yeast tRNA^{Phe} in a temperature range of 50 to 85 °C. These spectra were taken under following conditions: acquisition time, 1 s, 512 transients for spectrum a, 1024 transients for spectra b, c, d, and e.

midst or near the low end of the native-denatured transition, a considerable amount of broadening and shifting to high field takes place for all the resonances, though less for those from the Y base, At 40 °C only the resonances from the Y base, D and D′, m^2G_{10}, the T base and perhaps the m^5C are visible in the native tRNA^{Phe} spectrum measured with 0.01 M Mg^{2+}. The temperature transition profiles of the chemical shifts of these resonances are demonstrated in Figures 4 and 7.

The intact tRNA^{Phe} in the presence of Mg^{2+} was also investigated at 360-MHz frequency (Figure 10). In this sample, the internal standard, *tert*-butyl alcohol, was not added so that the spectral region near 1.1–1.4 ppm would not be masked by the signal from the *tert*-butyl alcohol. (The spectral position of the resonances were calibrated from the Y signal determined at 220 MHz; the chemical shifts of Y signal in the intact tRNA have been shown previously to be insensitive to temperature variation (Kan et al., 1974).) Due to a higher resolution power and sensitivity of the 360-MHz spectrometer, not only the signal-to-noise ratio of the spectra was improved but the low-field resonances of m^7G, m^1A, and Y″ now became much more evident (Figure 10). The spectral study at 360 MHz provides the following important results: (1) Comparing the nine spectra collected from 85 to 50 °C (five are shown in Figure 10), it is clear that line widths of several resonances, such as m^2G, D, m^5C, and T, are narrow at 85 °C (the denatured state), broad at intermediate temperature (69–75 °C), and then become narrow again at 50 °C (the native state, Figures 4 and 7). The broadness of these resonances at the transition temperature (75 °C) suggests the presence of multiple conformational states for each resonance at this temperature. In the approximation of a two-state transition, the product of the life time (τ) and

the chemical shift differences ($\Delta\nu$) between the two states for each resonance is $\approx 1/2\pi$ (2 to 2.5) (Pople et al., 1959). In other words, the transition has an intermediate exchange rate measured at 360 MHz at 75 °C. However, there is no obvious broadening effect on m^2G, D, and $m^2{}_2G$ methyl signals observed at 220-MHz spectrum at 75 °C (Figure 9c). This observation indicates that, when measured at 220 MHz, the exchange rate between the two states was sufficiently fast to avoid the broadening or splitting. (2) The dramatic shifting and splitting of the methyl resonance of T upon renaturation at low temperature now became much more obvious. After the initial broadening at 78 °C (Figure 10b), a small portion of T methyl signal appears at 1.0 ppm at 75 °C. This peak clearly becomes larger at temperatures below 69 °C, while the other portion of T could resonate at the same position of the two m^5C's (~1.55 ppm). Thus, the areas of the signal from C-CH_3 of Y base (~2.08 ppm), the two m^5C's (~1.55 ppm), and from T (~1.0 ppm) were integrated and compared at temperature below 60 °C. If the area of the well-resolved Y signal is taken as unity, then the area in the peak at 1.55 ppm and at 1.00 ppm is 2.4 ± 0.2 and 0.6 ± 0.2, respectively. This analysis supports the notion that, at the native state, the methyl group of T resonates both at 1.00 ppm (~60%) and at 1.55 ppm (~40%). (3) The signal at 3.03 ppm (50 °C or at the native state) is also evident in the 360-MHz spectrum (Figure 10e). Information from both studies at 220 MHz and 360 MHz suggests that this signal belongs to the D′ which clearly appears at 98 °C in Figure 9a or at 85 °C in Figure 10a and shifts progressively upfield when the temperature is lowered to ~80 °C (Figures 9b and 10b). During the temperature transition from 78 °C (Figure 10b) to 69 °C (Figure 10d), the D′ signal located at 3.5 ppm (75 °C) disappeared with the simultaneous emergence of a distinct peak at 3.03 ppm (69 °C). (4) The spectral data at 360 MHz provide additional information about the $m^2{}_2G$ signal. At 75 °C, the $m^2{}_2G$ resonance is broad in both 220- and 360-MHz spectra. At 69 and 50 °C, the spectra at 360 MHz (Figures 10d and 10e) provide fairly concrete information that the $m^2{}_2G$ resonance is a broad signal centered around 2.6–2.7 pm. (5) Much more useful information about m^1A is clearly visible with a narrow line width from 85 to 50 °C and with an upfield shift of less than 0.2 ppm from high to low temperature. As for the m^7G resonance, it is a sharp line at 85 °C and becomes broadened with a small upfield shift at 75 °C and then it is masked by the edge of the HDO peak which shifts slightly upfield at lower temperature. Though the m^7G resonance cannot be traced precisely at temperatures below 75 °C, it is unlikely that this signal is shifted dramatically upfield since all the upfield resonances at 50 °C can be adequately accounted for (Figure 10e). (6) Finally, when the intact $tRNA^{Phe}$ is in its native state (Figure 10e, 50 °C, with 0.01 Mg^{2+}), the following resonances are clearly evident in the 360-MHz spectrum: T (at two spectral positions), m^5C's, D (perhaps D′), m^2G, Cm, Gm, m^1A, Y, Y′, and Y″—resonances from a total of nine bases. The relatively narrow line widths of these resonances indicate that these methyl or methylene groups have a fair degree of motional freedom for their relaxation. The chemical-shift data of these groups from the nine bases should provide certain useful information about the conformation of the native intact $tRNA^{Phe}$ in solution. These chemical-shift data are presented in Figures 4, 5, and 7 and are tabulated in Table I.

Discussion

The studies of nucleic acids by NMR always should concern the phenomenon of *inter*molecular events vs. that of the *intra*molecular events, i.e., the problem of aggregation (Ts'o, 1974). Since the concentration of yeast $tRNA^{Phe}$ in this ^1H NMR study was about 10 mg/mL (Experimental Section), there is a possibility that the yeast $tRNA^{Phe}$ may aggregate at very low temperature in the presence of Mg^{2+}, a phenomenon which has never been reported in the literature or observed by us. However, the extensive broadening of methyl/methylene resonances at this condition could be an indication of aggregation. This phenomenon, therefore, necessitates the meaningful collection and discussion of the ^1H NMR data to be limited to the region of 45–100 °C, where the problem of aggregation is not expected to be serious and the resonances are well defined and well resolved. The problem for the aggregation of the fragments could be even more serious than that for the intact tRNA (Römer et al., 1970; Kearns, 1976). However, the concentrations of these fragments employed were considerably less (See Experimental Section) than the intact tRNA. In addition, these resonances from fragments, at appropriate temperatures, are well resolved, and their chemical shift values are similar to these from the intact tRNA. Thus, the ^1H NMR results reported here are interpreted on the basis of *intra*molecular effects, i.e., on the basis of the conformation of the fragments and the intact molecules. Such an interpretation should be valid under the conditions where the resonance lines are well defined and resolved.

(*A*) *The Effect of the Adding of Magnesium Ion and the Splitting of the Methyl Resonances of T Residue into Two Peaks at Low Temperature.* The transition profiles in Figures 4 and 7 show that the midpoints of these chemical shifts vs. temperature profiles are at higher temperatures in the presence than in the absence of magnesium ion. The average difference is about 20 °C. Furthermore, the breadth of these transition curves is also much narrower with Mg^{2+} in the solution. These results clearly indicate that Mg^{2+} greatly stabilizes the native form of yeast $tRNA^{Phe}$ in aqueous solution. The midpoints of these curves, or T_m, which belong to residues located in both stem regions (m^2G and two m^5C's) and single-stranded regions (two D's, m^1A, T, and $m^2{}_2G$), all are very close to about 74 °C. This finding suggests that secondary and tertiary structures of yeast $tRNA^{Phe}$ disintegrate simultaneously when heated in the presence of Mg^{2+} in solution. This conclusion is in agreement with the UV spectrometric studies on yeast $tRNA^{Phe}$ (Levy et al., 1972).

The observed phenomenon of splitting the methyl resonance of T base into two peaks at low temperature strongly suggests that the methyl group of T residue are in two magnetic environments (therefore, implies two conformations) in the native form of yeast $tRNA^{Phe}$ with Mg^{2+}. In this situation, the exchange rate between these two environments should be sufficiently slow ($\tau\Delta\nu \approx 10/2\pi$) in order for these two peaks to be resolved (Pople et al., 1959). The upfield signal of the splitting T-methyl resonance is much narrower than the downfield one, implying a greater motional freedom of this conformational state. The results from the two T containing fragments of yeast $tRNA^{Phe}$, 47–76 and 54–57, provide additional information on this point. The methyl resonance of the short fragment (54–57) shows neither chemical-shift changes nor broadening when the temperature is changed from high to low. This result suggests this short oligomer has only one conformer of T residue on the average at all temperatures. On the other hand, at low temperature, the methyl resonance of T base in fragment 47–76 does split into two peaks with Mg^{2+} present, as the intact yeast $tRNA^{Phe}$ (Figures 6, 9, 10). The studies on these fragments offer the following conclusions: First, at low temperature (<20 °C, Figure 4d), the conformation of fragment 47–76 at

the region containing the T residue could be similar to that in the intact yeast $tRNA^{Phe}$ molecule since the chemical-shift values of the methyl resonance from the fragment (<1.6 and ~1.0 ppm) are similar to those from the intact tRNA (~1.4 and ~1.0 ppm). Second, the condition in forming two conformers affecting T residue in the fragment and the tRNA involves only the primary and secondary structure of TψC stem and loop, and not other parts of the tRNA molecule. Third, the major shielding effect on the methyl group of T_{54} must come from the neighboring G_{53} base. These conclusions suggest that the splitting of the T-methyl resonances may be explained on the basis of different stacking modes of $G_{53}T_{54}$ segment. Since the difference in chemical shifts between the upfield and downfield signals of the splitting T-methyl resonances is only 0.5 ppm, and the maximum shielding effect of a guanine base at 3.4 Å distance is about 0.7 ppm (Giessner-Prettre et al., 1976), the splitting of the T-methyl resonance is explainable entirely on the change of $G_{53}T_{54}$ stacking. In this situation, the formation of two conformers involving T residue may involve only a local variation of the TψC stem and loop region.

Kastrup and Schmidt (1975) have reported previously the splitting of the T-methyl resonance of *E. coli* $tRNA^{Val}$ at low temperature with Mg^{2+}. At 73.5 °C, the methyl resonance of the T residue from *E. coli* $tRNA^{Val}$ was found to be at 1.76 ppm, nearly identical with that from yeast $tRNA^{Phe}$ (Table I). They reported "near 68°, it broadens severely and below that temperature, it becomes two broad peaks, one near 1.3 ppm and one near 1.7 ppm. Between 64 and 38°, the downfield peak becomes narrower and much less intense relative to that at 1.26 ppm." However, at temperature below 56 °C, a resonance peak appeared at 1.00 ppm and became sizable at 38 °C; this peak was not mentioned by Kastrup and Schmidt (1975). It is tempting to speculate that the methyl resonance of T in *E. coli* $tRNA^{Val}$ was also split into two spectral positions in the native conformation, i.e., ~1.3 and ~1.0 ppm, a situation very similar to that reported here for the yeast $tRNA^{Phe}$. Daniel and Cohn (1975, 1976) reported only one methyl resonance for the T base in their spectrum of *E. coli* $tRNA^{Met}_{f1}$, which is located at 1.00 ppm. There are two plausible explanations for this observation in comparison to the present results on yeast $tRNA^{Phe}$: (1) Since the sequence of the TψC arm and loop of *E. coli* $tRNA^{Met}_{f1}$ (UCGGTψCA) is different from that of yeast $tRNA^{Phe}$ (UGUGTψCG), the methyl group of T base of *E. coli* $tRNA^{Met}_{f1}$ in native conformation may not exist in two different magnetic environments; (2) since the spectrum was recorded at 37 °C, it is possible that the lower field methyl signal (at ~1.4 ppm) may already broaden to the baseline in view of the fact that the ~1.4-ppm methyl resonance from yeast $tRNA^{Phe}$ almost disappeared due to broadening at 48 °C.

(B) The Mg^{2+} Effect on the Line Widths at Half-Height ($\Delta\nu_{1/2}$) of the Methyl/Methylene Resonances in the Intact Yeast $tRNA^{Phe}$. The $\Delta\nu_{1/2}$ values of the methyl/methylene resonances from the intact $tRNA^{Phe}$ are difficult to determine accurately at low temperature, since these resonances become very broad below 40 °C and have a poor signal-to-noise ratio.

Therefore, only the $\Delta\nu_{1/2}$ values of the methyl/methylene resonances of $tRNA^{Phe}$ between 50 and 85 °C at 360 MHz are discussed. Qualitatively, the $\Delta\nu_{1/2}$'s can be divided into three categories according to the temperature effects.

The first category includes the methyl signals from Cm, Gm, and Y (four methyl groups). The six $\Delta\nu_{1/2}$ values in this category are independent of the temperature changes as shown in Figure 11a. These $\Delta\nu_{1/2}$'s are slightly smaller at 85 °C (~8 Hz) and then increase to about 10 Hz from 80 to 50 °C (Figures 10 and 11a). These resonances all belong to the minor bases in the anticodon loop. The invariant $\Delta\nu_{1/2}$ values of Cm, Gm, and Y with respect to temperature changes are evident and indicate that the anticodon loop protrudes from the tRNA and does not associate with other parts of the molecule.

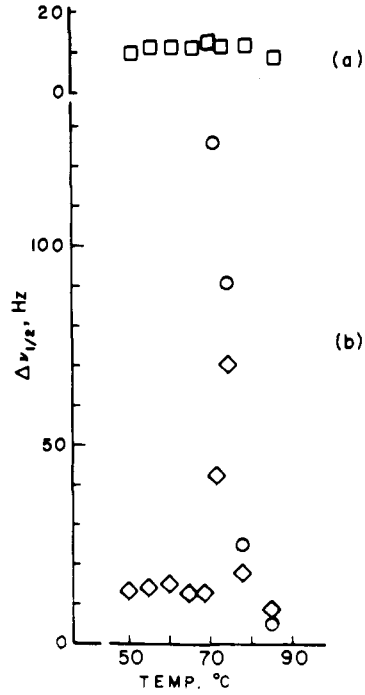

FIGURE 11: The line widths at half-height of methyl and methylene resonances vs. temperature profile. (a) The average $\Delta\nu_{1/2}$ values of Cm, Gm, and Y (four methyl groups); (b) ◇ represents the average $\Delta\nu_{1/2}$ values of two D's, two m^5C's, m^2G, T, and m^1A, and ○ represents the $\Delta\nu_{1/2}$ value of m^2_2G.

The second category contains the methyl resonances of m^2G, two m^5C's, T, and m^1A and the methylene resonances of two D's. The characteristic feature of this group is that the resonances are narrow at high temperature, becoming very broad at transition temperature (75 °C); and once again becoming narrow (~13 Hz) below the transition temperature. The entire process of variation in line width is completed within 10 °C (Figures 10 and 11b). This phenomenon reflects the abrupt nature of transition of $tRNA^{Phe}$ in the presence of Mg^{2+}. The residues which contribute these resonances are most likely involved in the secondary or/and tertiary structures of $tRNA^{Phe}$ in its native state. The broadening of these signals at the transition may represent the simultaneous existence of various conformational states.

The last category contains only one methyl resonance from m^2_2G. This resonance can be considered as an exception to the second category. The $\Delta\nu_{1/2}$ of m^2_2G is narrow at high temperature as well, broadening at transition temperature. However, it becomes even more broadened and finally disappears at lower temperature (Figures 10 and 11b), contrary to the resonances in the second category. Obviously, this residue is also involved in the secondary or tertiary structure of native $tRNA^{Phe}$. However, the broadening and disappearing of this resonance at low temperature may be caused by the immobility of the C_2–$N(CH_3)_2$ bond of the m^2_2G residue in the $tRNA^{Phe}$ molecule in its native state. Therefore, the $\Delta\nu_{1/2}$ of m^2_2G may reflect the restriction of rotation of the C_2–$N(CH_3)_2$ bond.

(C) The Comparison between the Observed and Calculated Chemical Shifts of Methyl/Methylene Resonances in Yeast

TABLE II: The Ring Current Anisotropic Shift Effect on the Methyl and Methylene Proton Resonances of a Yeast $tRNA^{Phe}$ Molecule in Crystalline State.[a]

Modified base	Shielded by	Ring current effect (ppm) 5-Membered ring	6-Membered ring	Sum	Obsd $\Delta\delta$ (ppm)
m^2G_{10}	C_{11}		0.03		
	U_{12}		0.01		
	C_{25}		−0.01	0.03	0.37
$D_{16,17}$ (C_5)	None			0	0.27
$D_{16,17}$ (C_6)	None			0	0.53
$m^2{}_2G_{26}$ (α)	m^2G_{10}	0.68	0.21		
	C_{11}	0.02			
	A_{44}	−0.02	−0.07	0.82	
$m^2{}_2G_{26}$ (β)	m^2G_{10}	0.13	-		0.46
	C_{27}		0.04		
	A_{44}	−0.04	-	0.13	
Cm_{32}	A_{31}	-	0.07		
	Y_{37}	−0.01	−0.01		
		Adduct ring −0.01[b]			
	A_{38}	−0.01	−0.08	−0.05	−0.08
Gm_{34}	U_{33}		0.01		
	A_{35}	−0.03	−0.03	−0.05	−0.13
Y_{37} (CCH_3)	A_{38}	-	~0		
	A_{39}	-	~0	0	0.08
Y_{37} (NCH_3)	A_{36}	-	0.1		
	A_{38}	0.05	-	0.15	0.17
Y_{37} ($COOCH_3(\alpha)$)	U_{33}		~0		
	A_{36}	−0.02	−0.03		
	A_{38}	-	+0.01	−0.04	−0.02
Y_{37} ($COOCH(\beta)$)	A_{36}	-	−0.03	−0.03	−0.01
m_5C_{40}	A_{29}	-	−0.02		
	G_{30}	−0.01	−0.02		
	A_{38}	0.10	0.16		
	ψ_{39}		0.12[c]	0.33	0.39
m^7G_{46}	A_9	-	0.09		
	A_{21}	0.11	0.02		
	G_{22}	−0.02	−0.01		
	G_{45}	0.04	-	0.21	<0.1
m^5C_{49}	U_6		0.02		
	U_7		0.10		
	U_{47}		~0		
	G_{65}	−0.02	−0.02		
	A_{67}		0.08	0.16	0.37
T_{54}	U_{52}		0.02		
	G_{53}	0.50	0.10		
	ψ_{55}		0.02[c]		
	A_{58}	−0.02	−0.03		
	A_{62}	-	0.08	0.67	0.39 and 0.87
m^1A_{58}	G_{18}	0.23	0.05		
	T_{54}		−0.01		
	C_{60}		0.04	0.31	0.15

[a] The atomic coordinates of yeast $tRNA^{Phe}$ are kindly provided by Dr. A. Rich, private communication. The graphic analyses of the ring current effect were from Giessner-Prettre et al. (1976). [b] By using the isoshielding contour of the five-membered ring of guanine base. [c] By using the isoshielding contour of uracil base.

$tRNA^{Phe}$. A comparison between the structure of tRNA and aqueous solution vs. that in crystal.

NMR data of the modified residues (1 to 4 ppm from DSS) and hydrogen-bonded NH resonances (11–15 ppm from DSS) can provide accurate information about the structure tRNA in aqueous solution. Such information, however, can be useful only when the NMR data can be analyzed in terms of a three-dimensional conformation; the conformation in aqueous

solution determined by NMR can then be compared with the conformation in crystal determined by x-ray diffraction. Any variance in the comparison can be due to a difference in structure at these two states (solution vs. crystal) or to inaccuracy of the conformation determination, or to both. At present, the NMR technique cannot be used to determine the tRNA conformation independently, but calculation of NMR data can be made from a given conformation. Therefore, the calculated NMR data were generated from the structure in the crystal determined by the x-ray diffraction method.

The calculated ring-current shielding/deshielding effects of the methyl or methylene groups from their neighboring bases in yeast $tRNA^{Phe}$ are listed in Table II. These calculations of shielding or deshielding effects were based on the structure of yeast $tRNA^{Phe}$ in orthorhombic crystal as described in atomic coordinates (A. Rich, private communication) and the graphic approach to the computation of the ring-current effects (Giessner-Prettre et al., 1976). A comparison between the calculated ring-current effect (in terms of ppm) is made to the observed shielding/deshielding effects which are defined by the differences in ppm of the chemical-shift values from the intact tRNA vs. these from the mononucleotides (/sides) determined at 40–50 °C, i.e., the lower plateau region with respect to the temperature perturbation (Table I).

The results in this comparison can be classified into four categories. The first category contains seven resonances, Cm_{32}, Gm_{34}, Y_{37} (all four methyl resonances), and m^5C_{40}. The observed and the calculated $\Delta\delta$ values of these seven resonances are in agreement with each other within 0.1 ppm. Since these four modified residues are either in anticodon loop or anticodon stem, the results suggest no difference in the conformations of anticodon stem and anticodon loop of this tRNA in aqueous solution vs. that in crystal can be found by this approach. In addition, this category also contains one unusual case which is $m^2{}_2G_{26}$. There are two ring-current effects predicted for the two methyl groups in $m^2{}_2G_{26}$ (0.82 and 0.13 ppm) based on a static structure of tRNA in crystal; but only one broad signal was seen on ^{1}H NMR spectrum at ~65 °C (Figures 9 and 10) having an observed $\Delta\delta$ value of 0.46 ppm. This observed value of $\Delta\delta$ is very close to the average of the two calculated values. As expected, the C_2–$N(CH_3)_2$ bond in $m^2{}_2G$ rotates with a sufficiently fast rate so that only one broad peak is observed. Therefore, it is possible that the average conformation of the two methyl groups from $m^2{}_2G$ in aqueous solution may also be no different from the average positions from its crystal form. Since $m^2{}_2G_{26}$ is located on top of anticodon stem, this reasoning again reinforces the preceding conclusion, i.e., the conformation of the anticodon region of yeast $tRNA^{Phe}$ in aqueous solution is similar to that in the crystalline state.

The second category contains m^2G_{10}, $D_{16,17}(C_5)$, $D_{16,17}(C_6)$, and m^5C_{49}. The observed $\Delta\delta$ of these resonances in this category are much higher (more than 0.1 ppm) than the calculated $\Delta\delta$, indicating these protons are *more* shielded than the predicted values based on crystal structure and the ring-current effects. While there exists some doubts about the assignment of $D_{16,17}(C_6)$ resonance in the native tRNA spectrum, this conclusion is most likely to be correct, since it is supported by the result on $D_{16,17}(C_5)$.

The third category contains m^7G_{46} and m^1A_{58}. The observed $\Delta\delta$ of these two resonances are much lower (0.15–0.2 ppm) than the calculated $\Delta\delta$, indicating these protons are *less* shielded than the predicted values based on crystal structure and the ring-current effects.

Finally, the fourth category contains T residue. The methyl resonance from T has only one predicted ring-current effect value but two $\Delta\delta$ values are observed (Table I). However, both experimental values are not in good agreement of the predicted value, interestingly, one was too high and the other too low.

In summary, the comparison indicates that no differences between observed and calculated $\Delta\delta$ values from resonances in the anticodon stem and loop can be found, but differences in TψC stem/loop and D stem/loop are uncovered. The nature of the results, with both agreement and disagreement involving too high and too low values, suggests that the difference may be indeed due to the difference in conformation. It should be noted that the regions showing the differences in comparison are the regions of the tertiary structure of the molecule which are more readily influenced by packing and also the areas which are less defined by the x-ray diffraction data. The segment involving $m^2{}_2G_{26}$ residue, which connects the D stem and anticodon stem, may have the same conformation in aqueous solution as in crystalline state except the C(2)–$N(CH_3)_2$ bond in $m^2{}_2G$ is rotatable in aqueous solution but fixed in the solid state. Currently, there is no high-field NMR data concerning the acceptor stem region, except one preliminary result on the yeast $tRNA^{Phe}$ with a phenylalanine attached at the CCA end through an amide linkage on A_{76}. It was found that the $-CH_2-$ resonance on phenylalanine is not broadened at low temperature (Kan, Ts'o, Sprinzl, and Cramer, unpublished data). This indicates that the CCA end of yeast $tRNA^{Phe}$ is not associated with any other part of the molecule in the native form.

The following paper of this series (Kan and Ts'o, 1977) concerns the hydrogen-bonded NH protons of yeast $tRNA^{Phe}$ and also indicates the same finding as in this paper, i.e., the conformation of yeast $tRNA^{Phe}$ in solution is grossly similar but not identical with that in the crystalline state, particularly in the areas of TψC region and D region.

Acknowledgment

We also thank Dr. Oleg Jardetzky for the use of the HX-360 MHz spectrometer located at Stanford University, Drs. Woodrow W. Conover, Stephen Patt, and Thomas Wagner for their excellent technical assistance, and Dr. A. Rich for providing us with the yeast $tRNA^{Phe}$ atomic Cartesian coordinates.

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Comparison of the Fine Specificity of Anti-Dinitrophenyl-Combining Site Composed of Either V_L Dimer or V_L and V_H of Protein 315†

Moshe Gavish, Raymond A. Dwek,‡ and David Givol*

ABSTRACT: The Fv fragment (Hochman, J., Inbar, D., and Givol, D. (1973), *Biochemistry 12,* 1130) derived from mouse myeloma protein 315, possessing anti-dinitrophenyl (Dnp) activity, is composed of the variable portions of light chain (V_L) and heavy chain (V_H) of the intact immunoglobulin. After dissociation of Fv in 8 M urea, V_L and V_H were isolated and analyzed for their hapten binding properties. V_H was found to be an aggregate without anti-Dnp binding activity, whereas V_L was a dimer with a molecular weight of 24 000 and possessed two binding sites for N^{ϵ}-2,4-dinitrophenyllysine with an association constant of 2.3×10^3 M^{-1}. The binding properties of V_L dimer were found to be identical with those of the light chain dimer of protein 315 previously reported (Schechter, I., Ziv, E., and Licht, A. (1976), *Biochemistry 15,* 2785) and exclude the constant part of light chain from participating in the combining site of L dimer. The fine specificity of the anti-Dnp binding site of V_L dimer (V_L-V_L) or L dimer (L-L) was compared with that of Fv (V_L-V_H), or of the intact protein (L-H) by analyzing the binding of a homologous series of Dnp ligands. The affinity of protein 315 for Dnp-lysine is approximately 1000-fold greater than that of V_L dimer, whereas for DnpOH the affinity of the intact protein is only 6.5-fold greater than that of V_L dimer. Thus the subsite for binding the Dnp ring per se can be localized within V_L. On the other hand, the interaction with the side chain of Dnp ligands is negligible in V_L dimer or L dimer, whereas it is very pronounced in the intact protein, suggesting that V_H contributes most of these interactions. The binding of "strange" cross-reacting haptens (Michaelides and Eisen, 1974) like dinitronaphthol and menadione was also localized within V_L dimer. Moreover the affinity of V_L dimer toward Dnp-lysine, dinitronaphthol, and menadione is similar, whereas the intact protein, or Fv, binds Dnp-lysine much better than they bind dinitronaphthol and menadione. Upon binding of dinitronaphthol or Dnp-caproate to V_L dimer, a red shift is observed in their absorbance spectrum. This red shift is similar, although not identical, to that observed with Fv and indicates that in either V_L dimer or Fv the ligands interact with a tryptophan residue. V_L dimer has only two tryptophan residues one of which (Trp-35L) is a constant residue present in the domain interior, whereas the other tryptophan is localized in the third hypervariable region of V_L (Trp-93L) and must therefore be the one interacting with the aromatic haptens. This assignment is in agreement with a model building study of protein 315 combining site (Padlan et al., 1977). The problems of antibody multispecificity and of subsites localized on different chains are discussed.

The Dnp binding site of the mouse myeloma protein 315 (Eisen et al., 1968) has been the subject of many investigations. Several parameters of the fine structure of this site were analyzed by affinity labeling (Goetzl and Metzger, 1970; Givol et al., 1971; Haimovich et al., 1972), binding of "strange" cross-reacting ligands (Michaelides and Eisen, 1974) by kinetic mapping with various ligands (Haselkorn et al., 1974), circular dichroism (Hochman et al., 1973; Freed et al., 1976), and magnetic resonance (Dwek et al., 1975a,b). These studies elucidated the dimensions of the site and the contribution of various side chains to the binding site. They also helped to divide the site into several subsites with hydrophobic or positively

† From the Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel (M.G. and D.G.), and the Department of Biochemistry, University of Oxford, Oxford, United Kingdom (R.A.D.). *Received January 18, 1977.* Supported in part by a grant from the Israel Commission for basic science.