^{1}H Nuclear Magnetic Resonance of Modified Bases of Valine Transfer Ribonucleic Acid (*Escherichia coli*). A Direct Monitor of Sequential Thermal Unfolding†

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ABSTRACT: Proton magnetic resonances at 220 MHz from three nucleotide residues of valine I tRNA (*Escherichia coli*) serve as intrinsic probes of local molecular structure. Resonances from the methyl group of ribothymidine, the methyl group of N^6-methyladenosine, and the C-5 methylene of dihydrouridine monitor separate conformational transitions in the TψC, anticodon, and dihydrouridine loops, respectively. As the temperature is raised in a solution containing 0.23 *M* Na^+ and no Mg^{2+}, the dihydrouridine region melts with a T_m of 55°, the anticodon region at 58°, and the TψC region at 67°. The dihydrouridine nuclear magnetic resonance (NMR) transition correlates with the major change in absorbance monitored in the uv at 330 nm which is ascribed to structural perturbations near the 4-thiouracil moiety. On the NMR time scale slow exchange is seen throughout the temperature range for dihydrouridine and below the apparent T_m for the ribothymidine methyl group. Chemical shift and line width differences between folded and unfolded forms of the polynucleotide indicate that, in the native structure, ribothymidine is in a highly structured region and N^6-methyladenosine is in a somewhat less restricted environment. Narrow line widths for the C-5 methylene triplet are found over the whole temperature range indicating that this base is undergoing rapid internal reorientation relative to the overall macromolecule.

The course of protein synthesis involves transfer RNA in a host of interactions with proteins and other nucleic acids. tRNA molecules probably respond to these manifold perturbations through conformational changes of several sorts. Investigations by thermodynamic (Cole et al., 1972; Brandts et al., 1974) and kinetic (Cole and Crothers, 1972; Riesner et al., 1973; Crothers et al., 1974) methods indicate that indeed tRNA molecules have a variety of conformational forms available to them. Extended forms with both secondary and tertiary structures different from the "native" form have been proposed to exist for several tRNA species under conditions of low salt and minimal Mg(II) ion concentration (Cole et al., 1972). Other more subtle changes in tertiary structure are found at higher salt concentrations when the temperature is moderately increased. These structural variants may well have importance in the mechanism of protein synthesis and thus merit further detailed examination.

We have been exploring the use of proton magnetic resonance for monitoring structural changes, particularly those occurring in single-stranded regions. Previous work has demonstrated that resonances from modified bases provide useful intrinsic probes of structure in their vicinity. We found that peaks from a modified base in the anticodon loop of $tRNA^{Tyr}_{coli}$ were narrow and well resolved at low temperatures while the ribothymidine methyl peak of the TψC loop was broad (Koehler and Schmidt, 1973). Kan et al. (1974) followed the peaks from modified bases in $tRNA^{Phe}_{yeast}$ over the temperature range 30–80°. They found large effects on chemical shifts and line widths for several bases and were able to monitor thermally induced unfolding of the dihydrouridine region and TψC loop.

We report here results with $tRNA^{Val}_I$ from *Escherichia coli*. The chemical shifts and line widths of proton resonances from dihydrouridine, N^6-methyladenosine, and 5-methyluridine were measured over the temperature range 28–77° for the tRNA in 0.23 *M* Na^+ without Mg^{2+}. Each modified base peak monitors a separate thermal transition; line widths and chemical shift changes for the folded conformation are markedly different for the three probes. Perhaps most intriguing, the dihydrouridine and ribothymidine resonances indicate that these groups exchange between magnetically distinct environments at rates which are unusually slow.

Experimental Section

$tRNA^{Val}_I$ was obtained from tRNA which had been isolated from *Escherichia coli* K12M72(44-1)F$^-$ strain harvested in mid-log phase.[1] Initial fractionation of 20 g (400,000 A_{260} units[2]) of the tRNA, using 2 DEAE-Sephadex A-50 column steps, was carried out according to the method of Miyazaki et al. (Miyazaki et al., 1966; Miyazaki and Takemura, 1966). Valine acceptor activity was determined by a modification (Kirkegaard, 1969) of the paper disk assay of Cherayil et al. (1968). Valine accepting frac-

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[1] The unfractionated tRNA was a gift of Professor Nelson Leonard. We thank Dr. Leslie Kirkegaard for his generous help in the fractionation procedure.

[2] Abbreviations used are: A_{260} unit, the amount of tRNA which when dissolved in 1.0 ml of buffer and measured with a 1-cm light path has an absorbance of 1.0 at 260 nm; M^6A, N^6-methyladenosine; T, ribothymidine; dhU, 5,6-dihydrouridine; DSS, 2,2-dimethyl-2-silapentanesulfonate; RPC-5, reverse phase chromatography using Plaskon beads (Allied Chemical Corporation) as the inert solid support and Adogen 464 (trialkylmethylammonium chloride in which the alkyl is primarily C_8–C_{10}) as the immobile organic phase.

tions from the second A-50 Sephadex column were pooled and precipitated with ethanol. Each $tRNA^{Val}$ fraction was further purified using a 6 × 100 cm RPC-5 column (Pearson et al., 1971), equilibrated at 37° with 0.01 *M* $MgCl_2$–0.02 *M* imidazole (0.01 *M* imidazole–0.01 *M* imidazole·HCl) (pH 7.0)–0.5 *M* KCl. The tRNA was eluted in a 0.5 *M* KCl to 0.9 *M* KCl gradient in the same buffer. All tRNA fractions which contained valine acceptor activity from this column were pooled and precipitated with ethanol. Repeated attempts at further purification using RPC-5, varying elution temperature, pH, and column load, were unsuccessful. The valine acceptor activity at this point was 530 pmol/A_{260} unit of tRNA (~30% of the value assumed for pure species). Final separation of $tRNA^{Val}_I$ from other tRNAs was achieved using benzoylated DEAE-cellulose as described by Nishimura (1971). Two peaks of valine acceptor activity were observed. The first peak, comprising ~80% of the valine-acceptor activity, was identified as $tRNA^{Val}_I$ in accordance with the results of Nishimura (1971). This peak contained 33% of the total 260-nm absorbance from the column.

Initial NMR spectra of the region between 1.4 and 3.6 ppm from DSS (2,2-dimethyl-2-silapentanesulfonate) showed several peaks which appeared to be those of cetyltrimethylammonium bromide, or Adogen 464 (trialkylmethylammonium chloride in which the alkyl is predominately C_8–C_{10}) from the RPC-5 resin. An attempt was made to separate the tRNA from these smaller contaminants using Sephadex G-25. A subsequent check of the NMR spectrum showed the contaminant peaks to be greatly reduced in intensity. Comparing the area of the methylammonium peaks to that of the N^6-methyladenosine yielded a molar ratio for tRNA to quaternary ammonium salt of approximately 8:1.

Prior to use in the NMR, the $tRNA^{Val}_I$ was twice dialyzed against a 500-fold excess of 0.005 *M* phosphate–1 m*M* EDTA (pH 7.0) and twice against a 500-fold excess of 0.005 *M* phosphate (pH 7.0). Buffer solutions were prepared in glass-distilled H_2O. The $tRNA^{Val}_I$ was then lyophilized, dissolved in 2 ml of 99.77% D_2O, again lyophilized, and dissolved in 100% D_2O to give a final tRNA concentration of 1250 A_{260} units/ml, 0.025 *M* phosphate. The total Na^+ concentration in the NMR samples was 0.23 *M*, 0.045 *M* greater than the minimum required as tRNA phosphate group counterions.

Spectra were taken on a Varian HR 220 spectrometer, using a C1024 time-averaging computer; 500 scans were collected for each spectrum. Peaks were referenced internally with respect to either 2 m*M* *p*-dioxane or 2 m*M* *tert*-butyl alcohol. All chemical shifts are reported in ppm from DSS.

A single NMR sample was used for most of the work reported here. Each spectrum (temperature) required approximately 1.5–2 hr to collect. Between runs the sample was kept at 25° or (overnight between sets of spectra) at 4°. The spectra were acquired in the order 28, 42.5, 52, 62.5, 77.5, 70.5, 64, 59.5, 56, 70, 73.5, 67.5, 38, and 47°. No degradation of the sample was apparent in the NMR over this sequence and no nonreversible effects were seen.

Uv Absorbance–Temperature Profiles. Absorbance changes were monitored at 260 and 330 nm in separate experiments. Stoppered cells of 1-cm path length were used and measurements were made with a Beckman ACTA III recording spectrophotometer. The temperature was increased at a rate of 0.36°/min and was monitored via a thermocouple implanted in the cell chamber.

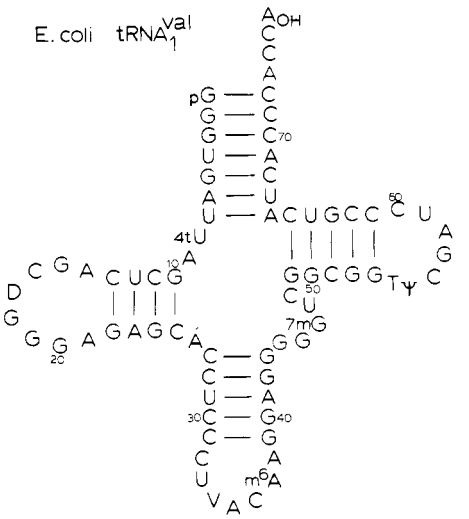

FIGURE 1: Nucleotide sequence for $tRNA^{Val}_I$ from *Escherichia coli* (Barrell and Clark, 1974).

Samples of $tRNA^{Val}_I$ from the NMR experiments were diluted into a buffer containing 0.025 *M* phosphate (pH 7.0) with enough added NaCl to bring the final Na^+ concentration to 0.15 *M*. The concentrations of tRNA used in the spectral measurements were 0.825 A_{260} unit/ml for the 260-nm experiment and 16.5 A_{260} units/ml for the 330-nm work. The absorbance measurements at 260 nm showed complete reversibility when the temperature was decreased from 90 to 25° over a time period of 110 min.

Results

Assignments of Peaks in High Temperature Spectra. As with all tRNAs the bulk of observable proton resonances of $tRNA^{Val}_I$ in D_2O fall in three major bands and provide little information. However, the region between 0 and 3.5–4.0 ppm from DSS contains only peaks from methyl and methylene protons of modified bases. The nucleotide sequence (Barrell and Clark, 1974) of $tRNA^{Val}_I$ is shown in Figure 1. Nucleosides expected to provide resonances in the high-field region are dihydrouridine (dhU) at position 17, N^6-methyladenosine (M^6A) at position 37, and 5-methyluridine (T) at position 54. 7-Methylguanosine (46), 5-oxyacetic acid uridine (34), and C-6 of dhU have resonances downfield of the high-field "window".

Figure 2 shows high-field proton spectra of $tRNA^{Val}_I$ at several temperatures. At high temperatures three major peaks are found between 0.5 and 3.5 ppm from DSS. The peak at 1.76 ppm at 73.5° is assigned to the methyl protons of 5-methyluridine (T) in the TψC arm. This assignment is based on spectra of the free nucleoside in D_2O and corresponds to that of Kan et al. (1974) for $tRNA^{Phe}_{yeast}$ at ~80°. The resonance at 2.68 ppm is an unresolved triplet ($J \simeq 7$ Hz) and is assigned to the C-5 methylene protons of the single dihydrouridine. For the free nucleoside in D_2O these methylene protons occur at 2.57 ppm from DSS; a triplet pattern is seen with $J = 7$ Hz. The peak at 2.84 ppm is assigned to the methyl protons of N^6-methyladenosine in the anticodon loop. While this nucleoside has not been examined in aqueous solution, in dimethyl sulfoxide the methyl peak is found at 2.55 ppm from internal Me_4Si (P. Sattsangi, personal communication). No other resonances from bases in $tRNA^{Val}_I$ are expected in this high-field region.

The small peak at 1.85 ppm is due to the methyl of residual acetate used in the buffer for the G-25 column. In the

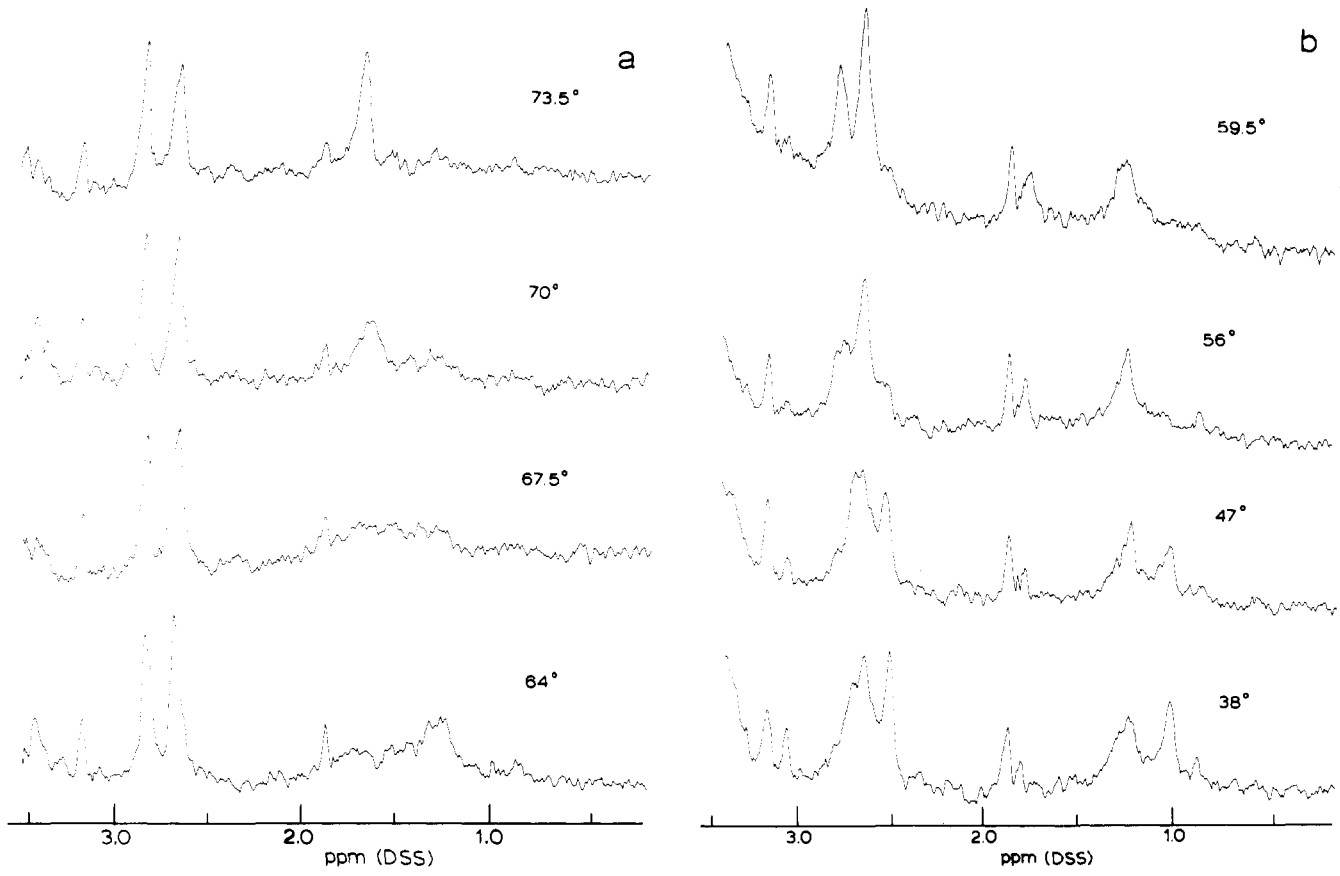

FIGURE 2: (a and b) High field 220-MHz spectra of $tRNA^{Val}_I$ in D_2O; 0.23 *M* Na^+–0.025 *M* phosphate, 1250 A_{260} units/ml of tRNA, pH 7. While dilute *p*-dioxane was used as an internal standard, the spectra are referenced to DSS (2,2-dimethyl-2-silapentanesulfonate); δ (*p*-dioxane) − δ (DSS) = 3.74 ppm. The probe temperature was controlled via a standard Varian unit. Sample temperatures were determined from the chemical shifts of ethylene glycol (Van Geet, 1968) measured before and after each run.

38° spectrum a very high field peak at 1.0 ppm and 2 peaks at 3.06 and 3.17 ppm are tentatively assigned to the cetyltrimethylammonium ion. As the sample is heated above 47° the singlet at 1.0 ppm broadens severely and disappears and the trimethylammonium peaks apparently coalesce to give a resonance at 3.15 ppm. This behavior is not fully understood at present but our observation of these peaks even after the extensive purification procedure used points out the tenacity of tRNA for certain solutes.

The areas of the resonances from M^6A, dhU, and T should be in the ratio of 3:2:3. The observed ratio is 3:3:3 within an estimated uncertainly of ±10%. Possible sources of this discrepancy include a heterogeneity for $tRNA^{Val}_I$ in its dhU content, under-methylation of the M^6A and T bases at positions 37 and 54 (the cells were harvested in mid-log phase), or contamination by other tRNA species. However, if another species were present at significant levels the areas of the T and M^6A peaks would not be the same since T occurs in virtually all tRNA and M^6A is quite rare.

Temperature Dependence of NMR Spectra. As the temperature is lowered from 74 to 38°, large changes occur in the three high-field resonances (Figure 2). Between 74 and 70° the T methyl peak moves upfield and broadens somewhat. 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. At 38° the line width of the peak at 1.26 ppm is ca. 40 Hz.

The dhU methylene behaves in quite different fashion. Between 65 and 28° the resonance at 2.68 ppm diminishes in intensity while a new peak grows at 2.5 ppm. Both resonances are fairly narrow over the whole temperature region. The peaks at 2.68 and 2.5 ppm represent dhU methylene protons in two magnetically distinct environments. The relative peak areas at each temperature are a measure of the distribution of dhU between those two environments.

For the M^6A methyl the situation is different yet. Between 70 and 40° the peak broadens progressively and moves upfield. This effect is most easily accounted for if the shift and line width at each temperature are weighted averages for high and low temperature conformations with rapid exchange between the forms.

These spectral effects are plotted in Figure 3. In line with the analysis of Cole et al. (1972) and Cole and Crothers (1972) we assume that, with 0.23 *M* Na^+, the tRNA at low temperatures (≤28°) is in its native conformation or a close variant. The NMR then monitors equilibria between this low temperature conformer and high temperature forms involving unfolded secondary and/or tertiary structure. For M^6A the fractional change in chemical shift of the methyl group is used to determine the population in the "folded" site. In the case of dhU the fraction of total peak area in the resonance at 2.5 ppm constitutes the measure of "folded" structure.

The data for T are not so easily converted to fraction "folded" and "unfolded". A simple model to explain the observed behavior involves two sites for the T methyl group:

$$a \underset{k_{ba}}{\overset{k_{ab}}{\rightleftharpoons}} b$$

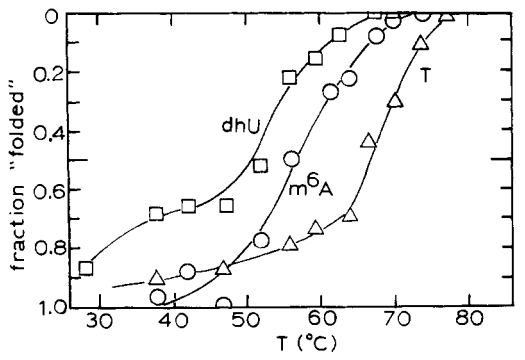

FIGURE 3: Fraction of dihydrouridine, N^6-methyladenosine, and ribothymidine groups in their respective "native" environments as a function of temperature. Chemical shifts and/or peak areas from Figure 2a and b were used for the calculation (see text).

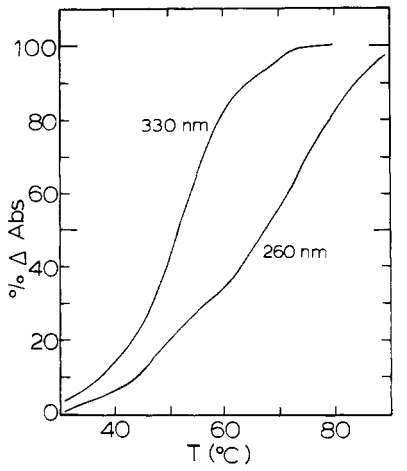

FIGURE 4: Absorbance-temperature profile for tRNA$^{Val}_I$ in 0.15 *M* Na^+ (pH 7.0). The absorbance was monitored at 260 nm (0.825 A_{260} unit/ml) and at 330 nm (16.5 A_{260} units/ml). The vertical scale represents the percent change in absorbance from 25 to 90° for the 260-nm run and 25 to 80° for the 330-nm data.

In site a the chemical shift (ω_a) is 1.8 ppm and the line width is narrow (<10 Hz). In site b the shift is 1.26 ppm and the line width is ca. 40 Hz (at 38°). Exchange between a and b is "rapid" above 70°.

If $\tau_{ab} = 1/k_{ab}$ and $\tau_{ba} = 1/k_{ba}$ then fast exchange obtains when $\tau = \tau_{ab}\tau_{ba}/(\tau_{ab} + \tau_{ba}) < |\Delta\omega|^{-1}$, where $\Delta\omega = \omega_a - \omega_b$ in rads/sec. Near 67° the exchange is "intermediate" ($\tau \approx |\Delta\omega|^{-1} = 1.3 \times 10^{-3}$ sec) and the populations of a and b are approximately equal. Below 60° the spectra exhibit behavior characteristic of slow exchange ($\tau \gg |\Delta\omega|^{-1}$). It appears that a narrow peak at 1.8 ppm diminishes in intensity at the expense of the broad peak at 1.26 ppm as the temperature is lowered to 38° (Figure 2b). But this conclusion is tenative because of the moderate signal-to-noise level.

A straightforward interpretation of the T methyl data can be made with site a representing the unfolded TψC arm and site b the modified base in the folded tRNA structure, where the methyl nuclei experience a large diamagnetic shielding (Kan et al., 1974).

In Figure 3 the fractional concentration of the T methyl group in site b vs. temperature is plotted. For 73.5 and 70° the exchange is rapid and the chemical shift represents an average weighted by the fractional populations in a and b. (The shift at 77.5° is assumed to represent 100% a.) At 64° and below the fractions in a or b are given by the relative areas of the peaks at 1.8 and 1.26 ppm, respectively.

The a to b equilibrium has a biphasic temperature dependence which would not be expected for a simple helix-to-coil transition of the TψC arm. Further work is under way to explore this unexpected observation and to extract rate constants and enthalpy changes from the NMR data (Beard, Kastrup and Schmidt, to be published).

Line Widths and Chemical Shifts at High and Low Temperatures. Line widths of the three resonances increase as the temperature decreases. The effect is greater for T; only a slight increase is found for dhU. At the highest and lowest temperatures chemical exchange does not contribute to the line widths which are instead determined by a combination of the overall rotational relaxation time of the macromolecule and the degree of internal motion of the modified bases themselves. Table I lists line widths and chemical shifts for the three groups at 77 and 38°.

Uv Heat Denaturation Curves. Absorbance temperature profiles monitored at 260 and 330 nm are shown in Figure 4. The transition seen at 330 nm is largely monophasic with a midpoint at approximately 50–52°. A second transition of smaller amplitude appears near 68°. At 260 nm the transition appears biphasic. An early transition is observed with a point of maximum slope near 47° and a later transition near 74°. Very similar results were obtained by Seno et al. (1969) for dialyzed tRNA^{Val} in 0.2 *M* NaCl with no added Mg(II).

Table I

	$\Delta\nu_{1/2}$ (Hz)[a]		δ (ppm, DSS)	
	77°	38°	77°	38°
Ribothymidine methyl	8 ± 2	40 ± 5	1.8	1.26
Dihydrouridine C-5 methylene	7[b] ± 3	10[b] ± 4	2.68	2.5
N^6-Methyladenosine methyl	7 ± 2	25 ± 5	2.84	2.7

[a] Uncorrected for broadening due to instrumental conditions. [b] For the individual lines of a triplet with 7 Hz coupling constant.

Discussion

Sequential Thermal Denaturation. Each modified observed base resonance of tRNA$^{Val}_I$ separately monitors structural reorganization in either the dihydrouridine loop, the anticodon loop, or the TψC loop. Apparently each spin system samples (at least) two magnetically distinct environments; the resonance positions and line widths reflect equilibria between low and high temperature conformations.

In the case of N^6-methyladenosine a single peak is observed whose shift and line width represent a fast exchange average between folded and unfolded forms. Both the line width and the chemical shift of this resonance change in concert through the temperature range in sigmoidal fashion. We conclude that the N^6-MeA methyl group is sensitive primarily to the helix–coil transition of the anticodon stem.

For dihydrouridine, the increase of intensity at 2.5 ppm coupled with a simultaneous decrease at 2.68 ppm as the temperature decreases is evidence of slow exchange between low and high temperature forms of the dihydrouridine loop.

The methyl resonance of T shows more complicated be-

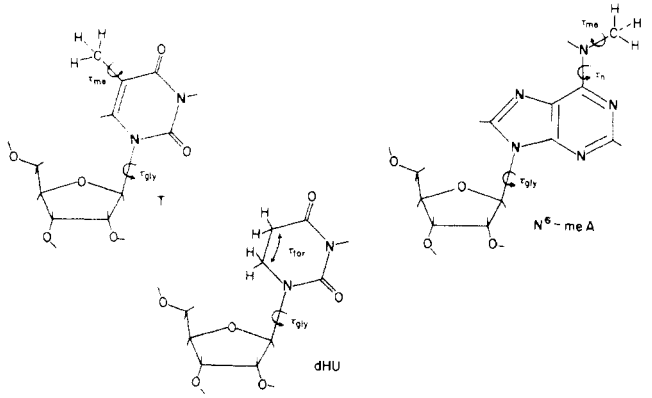

FIGURE 5: Structures of modified nucleosides observed in ^{1}H NMR spectra of tRNA$^{Val}_I$. Possible modes of internal reorientation are indicated.

havior. At temperatures up to ca. 60° there is probably slow exchange between a broad line at 1.26 ppm and a narrow peak at 1.8 ppm. Between 60 and 67° the resonances broaden markedly and coalesce. Above 70° a single resonance is found whose shift is a weighted average.

The thermal transitions for M^6A in the anticodon loop and T in the TψC loop are well separated with midpoints at 58 and 67°, respectively (Figure 3). In the case of dhU the transition appears to have a plateau. An early step, possibly representing tertiary structure changes, is complete at 40° and a later step of larger magnitude has a midpoint near 55°.

These results are in contrast to those of Kan et al. (1974) who examined tRNA$^{Phe}_{yeast}$ in similar experiments. With this species in low salt concentration (~0.04 *M* total cation, no Mg(II)) both the T methyl and dhU methylenes showed transitions with midpoints near 55°. The slow and intermediate exchange behavior found in the present work for tRNA$^{Val}_I$ was not observed for tRNA$^{Phe}_{yeast}$. Chemical shifts of methyl resonances from nucleosides in the anticodon loop of tRNA^{Phe} did not reflect conformational transitions in the range 30–80°.

Comparison of Uv and NMR Data. Absorbance at 330 nm of solutions of tRNA$^{Val}_I$ is largely due to 4-thiouridine at position 8 of the cloverleaf. The major change in A_{330} occurs with a midpoint of about 51° (Figure 4). This is very close to the midpoint of the major dhU NMR transition. It is thus likely that the A_{330} change correlates with structural changes in the dhU region.

A possible difficulty in comparing the NMR and uv data lies in the large concentration difference between the two measurements inasmuch as some tRNAs are known to aggregate at high concentrations under certain conditions. The problem was addressed by Crothers et al. (1974) who measured the absorption–temperature profile for formylmethionine tRNA (*Escherichia coli*) at concentrations of 12 and 1200 A_{260} units/ml. These measurements (at 335 nm) revealed no concentration dependence of the melting profile. While we have not made this direct comparison for valine tRNA there is no evidence to suggest that the high concentration used in the NMR work leads to significant aggregation. Indeed the resolved peaks are relatively narrow over the whole temperature range suggesting that the sample is substantially monomeric. It is therefore likely that valid comparisons can be made between the absorption–temperature profile and the NMR data.

The absorbance–temperature profile monitored at 260 nm is rather broad. About 20% of the absorbance increase occurs above 80° where the NMR indicates that structure in the TψC, dhU, and anticodon occurs has melted. Since, based on its sequence, the amino acid acceptor arm is expected to be somewhat more stable than the other stems (Gralla and Crothers, 1973; Uhlenbeck et al., 1974), the absorbance change above 80° may represent part of the unfolding of the acceptor stem.

Ribothymidine. While all three modified base resonances are sensitive to thermally induced conformational changes in the polynucleotide, there are marked differences in their behavior. The methyl resonance of T shows the largest change in chemical shift and line width on going from the unfolded to folded environment. From 77 to 38° the peak shifts 0.5 ppm upfield, almost surely as a result of positioning of the methyl group above the plane of another base (Kan et al., 1974). If the methyl protons are, on the average, about 3.4 Å or more from the plane of the shift-inducing base (or bases; a sandwich effect might be imagined) then the shift must come from a purine (or purines) (Giesser-Prettre and Pullman, 1970). The most likely candidate is the G at position 53 (next to T). G-57 and A-58 are also possibilities. However, other work will be required to define the stereochemistry in this region.

One interesting observation is that of Kim et al. (1974) and Robertus et al. (1974) who find that in crystals of tRNA$^{Phe}_{yeast}$ the T is probably involved in a base pair with the 1-methyladenosine located in the position corresponding to A-58 of valine (I) tRNA (*Escherichia coli*). If such a structure exists for the latter tRNA then that A should *not* be a source of the upfield shift for the T methyl because the two bases would be approximately coplanar, and the methyl group would be located in the plane of and away from the adenine ring.

The line width of the T methyl peak in the "native" form is 40 Hz at 38°. If nuclear relaxation is dominated by dipole–dipole interactions among the three methyl protons, then a line width ($\Delta\nu = 1/\pi T_2$) can be predicted from eq 1 assuming a rotational correlation time, τ_R, for overall motion of the macromolecule:

$$\pi\Delta\nu_{pred} = \frac{1}{T_{2pred}} = N\frac{3}{40}\frac{\hbar^2\gamma_H^4}{r^6}\left[6\tau_R + \frac{10\tau_R}{1+(\omega_0\tau_R)^2} + \frac{4\tau_R}{1+4(\omega_0\tau_R)^2}\right] \quad (1)$$

where N is the number of nuclei relaxing each proton (N = 2 for a methyl group), γ_H is the proton magnetogyric ratio, r is the internuclear distance for protons of a methyl group (taken as 1.78 Å), and ω_0 is the NMR resonance frequency ($2\pi 220 \times 10^6$ rads/sec).

Tao et al. (1970) followed the decay of fluorescence polarization anisotropy for ethidium bromide bound to tRNA. They measured an overall rotational reorientation time for tRNA$^{Phe}_{yeast}$ of 25 nsec at 25°. Komoroski and Allerhand (1972) obtained C-13 relaxation spectra of unfractionated tRNA from yeast. They calculated a value of about 30 nsec for τ_R between 35 and 54°. Using $\tau_R \simeq 25$ nsec, $\Delta\nu_{pred} \simeq$ 140 Hz. It should be noted that the dispersion terms of eq 1 (those containing a dependence on ω_0) contribute less than 5% to the calculated value. Thus $\Delta\nu$ depends directly on τ_R.

The observed line width for T (40 Hz) is much less than that predicted on the basis of overall rotation. This sort of behavior is often found, for example, in proteins where internal rotation of amino acid side chains decreases their nu-

clear relaxation rates. In general the decrease in line width depends on the value of the rotational correlation time for internal reorientation, τ_{int}, and the geometry. However, in the limit $\tau_{int} \ll \tau_R$ the relaxation rate becomes approximately independent of τ_{int} and reduces to

$$\frac{1}{T_2} = \left(\frac{3\cos^2\theta - 1}{2}\right)^2 \frac{1}{T_{2_{pred}}} \quad (2)$$

where $1/T_{2_{pred}}$ is the relaxation rate assuming no internal rotation (eq 1) and θ is the angle between the axis of rotation and the internuclear vector.

Referring to Figure 5, τ_{Me} is the correlation time for diffusional motion (or jumps between potential minima) around the methyl group axis and τ_{Gly} corresponds to rotation about the glycosidic bond. Other internal rotation modes are possible involving the sugar–phosphate bonds. Examining the effects of each mode in turn we find that rapid internal reorientation of the methyl group about its axis ($\tau_{Me} \ll \tau_R$, $\theta = 90°$) would reduce the predicted line width to 35 Hz. Further rapid motion about the glycosidic bond ($\tau_{Gly} \ll \tau_R$; $\theta \simeq 120°$) would have a marked effect, decreasing the line width to less than 3 Hz. Even if the methyl group were not rotating rapidly ($\tau_{Me} \gtrsim \tau_R$), if τ_{Gly} were small the line width would be less than 10 Hz. However, it is highly unlikely for methyl rotation to be restricted if other modes of motion in T are available. We conclude that the observed width of 40 Hz corresponds to rapid reorientation of the methyl group about its axis and restricted rotation for the other possible modes.

Dihydrouridine C-5 Methylene. In marked contrast to the T methyl, the dhU methylene resonance shifts only 0.18 ppm upfield and the line-width change is negligible when the temperature is lowered from 77 to 38°. The chemical shift change is in the direction predicted for a diamagnetic ring current effect, but the magnitude is too small to provide evidence as to which base is responsible for it. The following discussion suggests that the observed shift represents an average over several conformations.

It is particularly interesting to find that the C-5 methylene line width is so narrow in the folded tRNA conformation. If the base were immobile in the native form the peak width would be 70 Hz. Rapid internal rotation around the glycosidic bond ($\tau_{gly} < 10^{-9}$ sec) reduce this to less than 10 Hz for $\theta = 120 \pm 10°$. In their ^{13}C spectra of unfractionated yeast tRNA Komoroski and Allerhand (1974) noted that the dhU C-5 methylene carbon line was quite narrow (13 Hz), a result only consistent with fast internal rotation of the dhU rings of folded tRNA.

One other mode of internal motion should be considered here. It has been noted that the pseudotriplet pattern for the C-5 methylene of the dhU nucleoside results from torsional motions in the six-membered unsaturated ring (Deslauriers et al., 1971). This motion averages the chemical shifts for the two C-5 methylene protons and the coupling constants between the C-6 and C-5 protons sufficiently rapidly to give the triplet ($J = 7$ Hz). The simple spectrum persists in the tRNA at high temperature and (unresolved) at low temperature as well. But relatively slow torsional motion ($\tau_{tor} \lesssim 10^{-2}$ sec) is sufficient to average the spectrum to a triplet while $\tau_{tor} \lesssim 10^{-9}$ sec would be required to markedly affect the relaxation rate. In fact it is not clear that torsional motion of any rate would lead to reduced line widths. Thus the ring conformational fluctuation can probably not be looked to for the source of the extremely narrow dhU C-5 methylene line.

N^6-Methyladenosine. For N^6-methyladenosine the upfield shift in the low temperature spectrum is 0.14 ppm. While the methyl group is not situated in a strongly diamagnetic region, the shift clearly reflects a different environment for the spin system between unfolded and folded conformations.

The line width at 38° is 25 Hz. As with the ribothymidine methyl we can examine possible modes of internal rotation for their effect on the relaxation rate (see Figure 5). Fast rotation around the N-CH_3 axis ($\tau_{Me} \ll \tau_R$) would reduce the width to 35 Hz, close to the observed value of 25 Hz. Rotation about the C6–N6 bond has been studied for the base alone in organic solvents (Engel and von Hippel, 1974). Motion is highly restricted ($\tau_n \sim 10^{-3}$ sec at 20°) due to partial unsaturation of the bond; no line narrowing would be expected for such slow reorientation. Rapid rotation about the glycosidic and phosphodiester bonds would probably lead to a line width significantly narrower than that observed.

It is noteworthy that the *N*-methyl group is sensitive to its local conformation. In contrast, methyl groups of the Y base in $tRNA^{Phe}$ from yeast are apparently not strongly shifted between the native and denatured forms (Kan et al., 1974) perhaps because the bulky Y base side chain protrudes from the anticodon loop. The *N*-methyl, on the other hand, may be small enough to fit in the loop or at least to be shielded by the ring current of a nearby base. Further work is planned to utilize the M^6A methyl shift and line width as a probe of tertiary structure in the anticodon loop.

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Kinetic Investigation of Unfolding and Partial Refolding of a Crab Satellite $(dA\text{-}dT)_n$†

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ABSTRACT: Crab $(dA\text{-}dT)_n$ was isolated from the testes of *Cancer borealis* by a procedure involving separation of DNA and segregation of the satellite fraction by Hg^{2+} binding/Cs_2SO_4 density gradient ultracentrifugation. The titration of crab $(dA\text{-}dT)_n$ samples at 10° indicated a sharp absorbance change at pH 11.98 in agreement with the pH_m value observed for synthetic poly(dA-dT) under identical conditions. The reversal of the titration, however, resulted only in about 50% recovery of the original absorbance (at 260 nm) in marked contrast to the complete reversibility of the synthetic material. pH-jump experiments were carried out for the purpose of characterizing the rates and mechanisms of conformational transitions brought about by changes in the solution environment. It was found that the disintegration of the putative native structure of crab $(dA\text{-}dT)_n$ starts with a very fast reaction (occurring within the 6-msec deadtime of the instrument and comprising 65% of the total absorbance change) and it is completed via a slower first-order reaction (k = 66 sec^{-1}). It is postulated that the first process is due to the rapid untwisting of end regions and, perhaps, some short hairpin-like helical branches present on the macromolecules. The second reaction is believed to be the end-to-end type unwinding of the double-helical backbone of crab $(dA\text{-}dT)_n$. In the presence of low concentration (3 μg/ml) of Hg^{2+} ions the overall rate of disintegration process decreased drastically. pH jumps from pH values above pH_m to values below were used to study the rates of absorbance changes corresponding to the refolding of the strands of denatured crab $(dA\text{-}dT)_n$. A concentration independent process consisting of two phases was observed. The first phase was a gradual nonexponential process spanning the first second of the reaction, and the other, a very slow first-order process characterized by the rate constant value of 0.053 sec^{-1}. It is proposed that the first part of the process (involving about 24% of nucleotide residues) is an intramolecular formation of helical hairpins (frequently interrupted by mismatching bases) and the second part is a manifestation of some association of the extant unpaired bases during the folding of the branched structure. Refolded crab $(dA\text{-}dT)_n$ samples when subjected again to pH > pH_m in the stopped-flow apparatus displayed not the disintegration pattern of the native crab $(dA\text{-}dT)_n$ but rather that of synthetic poly(dA-dT). The marked facility of crab $(dA\text{-}dT)_n$ macromolecules for rapid conformational transitions induced by slight changes in the solution environment might be relevant to the biological function of this DNA.

Crab $(dA\text{-}dT)_n$ is the general name for various light satellite DNA preparations isolated from crab species (Sueoka, 1961; Widholm and Bonner, 1966; Pochon et al., 1966; Skinner, 1967; Skinner et al., 1970; Skinner and Kerr, 1971; Davidson et al., 1965; Brzezinski et al., 1969; Laskowski, 1972; Sabeur et al., 1969; Ehrlich et al., 1973). The distinguishing feature of these DNAs is their unusual primary structure which is almost entirely a strictly alternating sequence of A[1] and T nucleotide residues (Laskowski, 1972). Base composition and nearest neighbor frequency studies have indicated (Swartz et al., 1962; Laskowski, 1972) that in crab $(dA\text{-}dT)_n$ approximately 93% of the bases are alternating A and T, 4% irregularly occurring A and T, and 3% G and C, with moderate variations among different crab species. Whether the deviations from the al-

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[1] Abbreviations used are: A, deoxyadenylic acid residue or adenine base; T, deoxythymidylic acid residue or thymine base; G, deoxyguanylic acid residue; C, deoxycytidylic acid residue; SSC, 0.15 *M* NaCl-0.015 *M* sodium citrate buffer; 0.1 SSC, 0.015 *M* NaCl-0.0015 *M* sodium citrate buffer; A_{260}, absorbancy at 260 nm; EDTA, ethylenediaminetetraacetic acid; pH_i and pH_f, the initial and final pH values in a pH jump experiment.