A Study of Secondary and Tertiary Solution Structure of Yeast tRNA^{Asp} by Nuclear Magnetic Resonance. Assignment of G·U Ring NH and Hydrogen-Bonded Base Pair Proton Resonances†

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ABSTRACT: The 270-MHz spectra of yeast tRNA^{Asp} in H_2O solutions containing Mg^{2+} show clearly resolved resonances in the region from −15 to −9.5 ppm. Resonances between −15 and −11.5 ppm from the hydrogen-bonded protons of the acceptor stem and anticodon arm decrease in intensity with increasing temperature and disappear by 75 °C. Simultaneously, four well-resolved resonances between −11.2 and −10.3 ppm also decrease in intensity and disappear. Because of this behavior and their positions these resonances have been assigned to the four ring NH protons of G·U base pairs 5 and 30 in the acceptor stem and anticodon arm which are thereby shown not to be hydrogen bonded by normal Watson-Crick hydrogen bonds. The five G·C base pair resonances of the TψC arm remain visible above 70 °C after all other resonances have disappeared. The high-temperature tRNA spectrum agrees well with that of the isolated TψC hairpin and CCA half-molecule fragments, each of which contains the same five hydrogen-bonded proton resonances. The root-mean-square error between the observed and calculated resonance positions for the hydrogen-bonded base pair protons of these three arms is 0.19 ppm. The dihydrouridine stem is expected to have two A·U Watson-Crick base pairs and no G·C base pairs. However, it does not contribute any hydrogen-bonded resonances to the nuclear magnetic resonance (NMR) spectrum below −11.5 ppm. This suggests that even at 35 °C this helix is not hydrogen bonded in a normal manner. In the region below −11.4 ppm there are three additional proton resonances melting earlier than the rest which cannot be assigned to a particular helix of the cloverleaf. We suggest that these resonances arise from hydrogen-bonded protons involved in stabilizing tertiary structure.

Transfer RNA molecules are a class of biological macromolecules which show an interesting property in their secondary structure. Every molecule whose sequence has been determined can be folded into one or more variations of the basic cloverleaf structure which was postulated by Holley et al. (1966). More recent studies on several tRNA species using high-resolution nuclear magnetic resonance (NMR) (Shulman et al., 1973) are consistent with this secondary structure as are the x-ray crystallographic studies on yeast tRNA^{Phe} (Kim et al., 1974; Robertus et al., 1974). Whether or not these similarities will carry through for other species of tRNA with the same function or for tRNA molecules with functions other than protein synthesis (i.e., gene regulation, cell wall synthesis) will only be known after further solution and x-ray structure studies.

Even within a given tRNA molecule there is a very high degree of structural regularity uncommon to most biological macromolecules. This has become clearly evident in the past few years from the high-resolution NMR studies on these molecules. When the ring current calculations for various nucleotide bases (Giessner-Prettre and Pullman, 1970) were combined with diagrams of Arnott (1971) predicting the overlap of bases in an RNA double helical structure, Shulman et al. (1973) devised a table of ring current shift parameters which enabled them to predict NMR positions for hydrogen-bonded Watson-Crick base pair protons. To a first approximation these chemical-shift positions are dominated simply by the nature of the base pair being observed and that of the first adjacent base pair above and below in the double helix. Considering that these chemical-shift predictions assume perfect helicity and are quite sensitive to slight structural changes, it is remarkable that the root-mean-square error between observed and calculated resonance positions for some 35 Watson-Crick base pair hydrogen-bonded protons in two tRNA molecules, *Escherichia coli* tRNA^{Glu} and tRNA^{Gly}, is less than 0.17 ppm (Hilbers and Shulman, 1974; Hilbers et al., 1976). Again in this present study a similar level of agreement is observed for 16 out of 17 of the secondary structure "base pair" proton resonances which can be identified.

In the NMR studies which have been reported to date all of the secondary structure hydrogen-bonded proton resonances have been located in the region between −15 and −11.5 ppm. However, spectra of each individual species have also contained exchangeable proton resonances in the region −11.5 to −9 ppm. It has been suggested, on the basis of their chemical-shift positions, that some belong to non-hydrogen-bonded ring NH protons (Wong et al., 1972) and others to hydrogen-bonded exocyclic amino groups (Reid et

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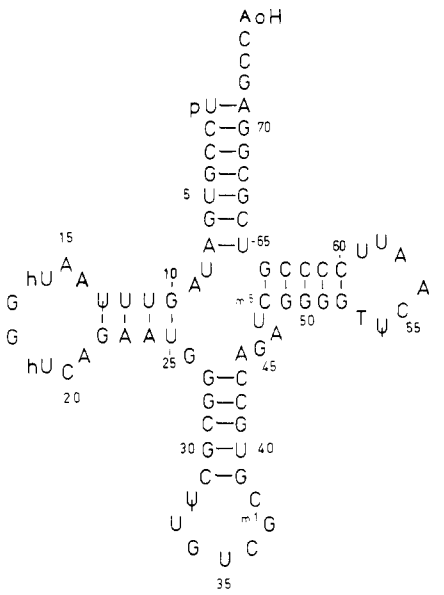

FIGURE 1: The Cloverleaf sequence of yeast $tRNA^{Asp}$ as determined by Keith et al. (1970) and Gangloff et al. (1971).

Table I

Base Pair No.	Calcd Position (ppm)	Exptl Position (ppm)	Calcd − Obsd
7	−14.50	−14.50	0
1	−14.00	−13.64	−0.36
31	−13.50	−13.64	+0.14
52	−13.35	−12.90	−0.45
28	−13.25	−13.24	−0.01
48	−12.90	−13.15	+0.25
4	−12.90	−12.97	+0.07
29	−12.80	−12.94	+0.14
51	−12.75	−12.60	−0.15
50	−12.75	−12.79	+0.04
49	−12.30	−12.22	−0.08
3	−12.30	−12.34	+0.04
2	−12.15	−12.30	+0.15
27	−13.0 to −11.8 (1.2 ppm for A-44)	−12.54	
6	−11.70	−11.63	−0.07

al., 1975). In this report we analyze the low-field proton NMR spectra of yeast $tRNA^{Asp}$ in which we can clearly distinguish four resonances between −10.2 and −11.3 ppm whose temperature dependence allows us to assign them to slowly exchanging ring NH resonances of G·U "base pairs" 5 and 30. In combination with NMR studies on the purified CCA half-molecules we have also been able to assign all of the secondary structure hydrogen-bonded proton resonances.

Recent studies demonstrate one extra proton resonance in *E. coli* $tRNA_2^{Glu}$ between −15 and −12 ppm which cannot be assigned to the cloverleaf (Hilbers and Shulman, 1974) and approximately six extra resonances in the −11.5- to −15-ppm region in *E. coli* $tRNA_1^{Val}$ (Reid et al., 1975; Reid and Robillard, 1975). This later observation is in quite good agreement with the extra base pairs seen in the crystal structure of yeast $tRNA^{Phe}$ (Kim et al., 1974; Robertus et al., 1974). Such extra base pairing is again seen in the present study in which three resonances below −11 ppm have been assigned to tertiary structure interactions.

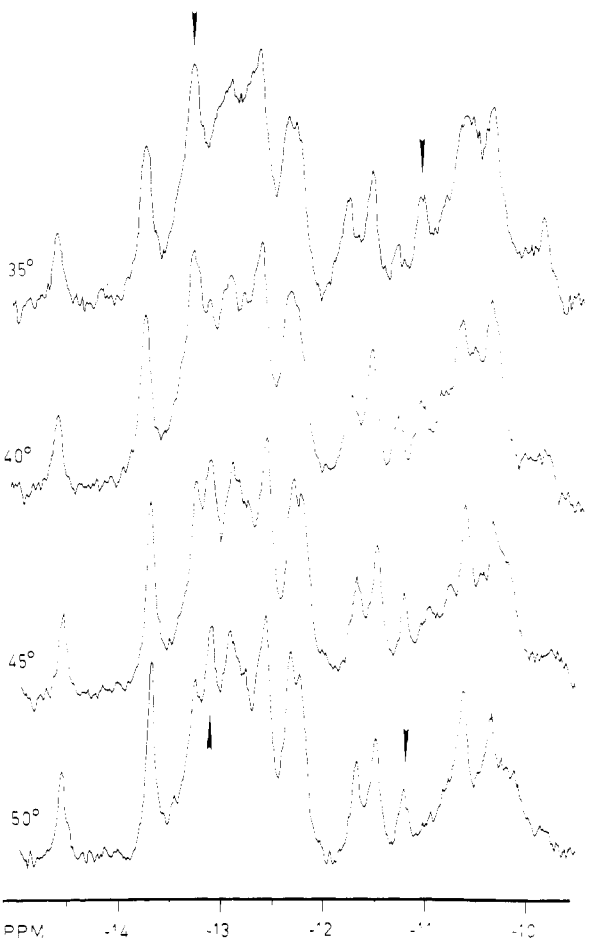

FIGURE 2: Proton NMR spectra (270 MHz) of yeast $tRNA^{Asp}$ between 35 and 50 °C. The buffer conditions and sample concentration are given in the Materials and Methods section of this paper. All spectra of the intact molecule presented in this and subsequent figures were taken on the same sample during the same day. Each spectrum presented represents the exact same number of accumulations with all instrument settings the same. All spectra were taken with a sweep rate of 36 Hz/s. The arrows indicate the starting and ending positions of the two proton resonances which change position as a function of temperature.

Materials and Methods

Yeast $tRNA^{Asp}$ and the fragments of the CCA half-molecule and the TψC hairpin were prepared as described previously (Coutts et al., 1974). All NMR samples were 1–2 mM tRNA, 1 mM EDTA, 10 mM sodium cacodylate (pH 7), and 15 mM $MgCl_2$.

The NMR spectra were taken by continuous wave accumulation on a Bruker 270-MHz spectrometer operating in the frequency sweep mode. The instrument was field locked on the H_2O resonance from the solvent. The temperature was controlled to ±1 °C. All chemical-shift positions are given in parts per million (ppm) relative to DSS (sodium 4,4-dimethyl-4-silapentanesulfonate). The NMR spectra simulation was carried out on a CDC 7600 using a program which generates a Lorentz convolution of a set of sticks of a given total area, position, and line width. The normalization is based on the specified height of a one-proton line with a given line width at half-height.

Results and Discussion

Sequential Melting. The cloverleaf sequence of yeast $tRNA^{Asp}$ is presented in Figure 1. From this sequence and

the ring current shift parameters tabulated by Shulman et al. (1973) the resonance positions of the Watson-Crick base pair protons have been calculated and are presented in Table I and at the bottom of Figure 3.

The NMR spectra of yeast tRNA^{Asp} were measured from 25 to 75 °C at intervals of 5 °C. At 25 and 30 °C the spectra were broad and poorly resolved presumably due to intermolecular association at the high concentrations necessary for these experiments. At 35 °C and higher the lines are considerably narrower but there is no change in overall intensity between 25 and 35 °C. From 35 to 50 °C there is also no decrease in the integrated intensity in the region −15 to −11.5 ppm (Figure 2). However, there is a shift of a resonance from −13.2 ppm to −13.08 ppm as indicated by the arrows at the top and bottom of the figure. It is in the slow exchange limit on the NMR time scale as indicated by the gradual decrease, without broadening, of the intensity at −13.2 ppm and the complementary increase in intensity at −13.08 ppm between 40 and 50 °C. There are simultaneous changes in the intensity in the high-field region; a new resonance appears at −11.16 ppm while there is a substantial decrease in intensity between −11 and −10 ppm. Again this appears to be a slow exchange of a single proton between two states. The shift of these two resonances apparently reflects some temperature-dependent conformational change in the intact molecule.

The first net loss of intensity which occurs can be seen in Figure 3. Between 50 and 65 °C there is a clear loss of three resonances (cf. arrows in 50 °C spectra). The first, at −13.08 ppm, is the same resonance that shifted between 35 and 50 °C (Figure 2). A second resonance disappears at −12.64 ppm as indicated by the decrease of intensity of this resonance and a deepening of the trough adjacent to it at −12.7 ppm (this can also be seen from the spectrum simulation in Figure 6). A third resonance at −11.45 ppm disappears at about the same time. Since the DHU arm with only two A·U base pairs is the least stable of the four arms, it would be expected to melt early (Gralla and Crothers, 1973). However, examination of the predicted resonance positions for this arm (bottom of Figure 3) suggests that the three resonances lost between 50 and 65 °C do not originate from the DHU arm. Since we are able to account for all base pair hydrogen-bonded protons in the other three arms by the remaining resonances (cf. next section), these three extra proton resonances must be associated with tertiary structure hydrogen bonds.

The importance of this result is that if the three early melting resonances cannot be assigned to the DHU arm and all other resonances are assigned to base pairs in the acceptor stem, anticodon, and TψC arms, then there are no resonances below −11.5 ppm for the base pairs of the DHU arm. Therefore, either the arm is not stabilized by normal Watson-Crick type hydrogen bonds, or the hydrogen-bonded protons of this arm are in fast exchange with solvent protons.

After these three distinct losses, there is a gradual decrease in the intensity of other resonance peaks between 60 and 70 °C (Figure 3), suggesting that part of the molecule is beginning to melt slowly. However, it is obvious that each individual arm does not melt separately so that sequential melting alone will not be sufficient for unambiguous assignments.

Assignments from Fragment Studies. The studies of Coutts et al. (1974) demonstrate that the TψC arm is, by far, the most thermodynamically stable of the four helical

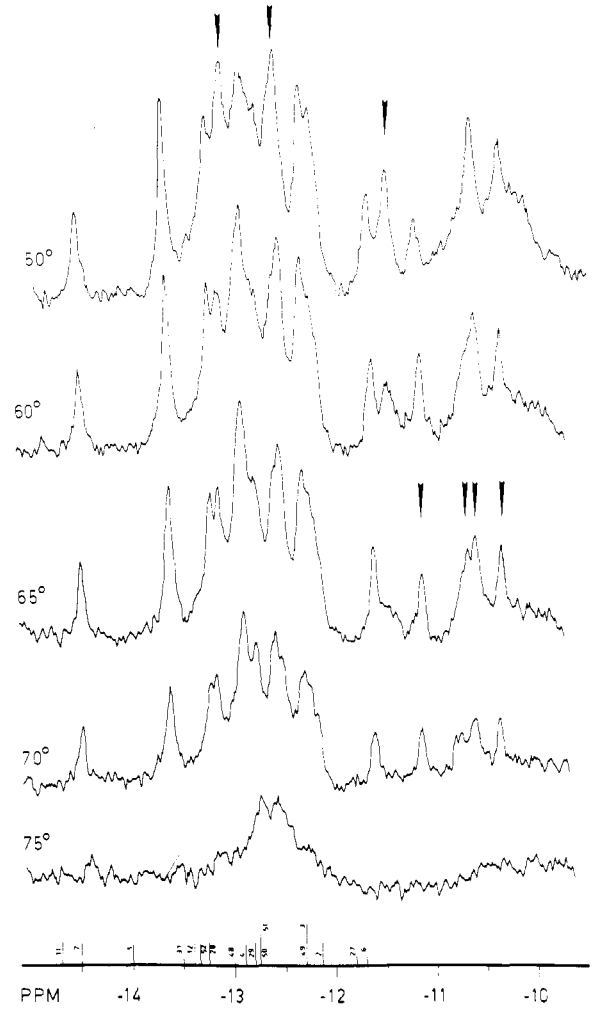

FIGURE 3: Proton NMR spectra (270 MHz) showing the 50 to 75 °C temperature dependence of tRNA^{Asp}. Details of the measurements are the same as in Figure 2. The three lowest field arrows indicate the positions of the three resonances which melt discretely between 50 and 65 °C. The four upfield arrows designate the positions of the G·U ring NH proton resonances. The positions marked at the bottom of the figure indicate the positions of the hydrogen-bonded base pair protons in the helical regions predicted on the basis of ring current shift calculations (Shulman et al., 1973).

regions in the intact molecule, having an optical T_m above 90 °C in 0.5 M NaCl solutions. The same behavior was observed for this helical region in the isolated CCA half-molecule. As a starting point for assigning the resonances in the intact molecule, we have examined the TψC helix in the isolated CCA half-molecule assuming that, since it is the most stable helix in the molecule, it might be the last to melt in the intact structure. Its behavior as a function of temperature is shown in Figure 4, spectra A and B. At the bottom of the figure we have marked the resonance positions for the individual base pair hydrogen-bonded protons predicted from ring current shift calculations (Shulman et al., 1973). The only calculated position which does not agree with the experimental spectra is that of G·C-52 which was calculated assuming only a slight upfield shift from T-53 and U-59 in the loop region. However, other stacking arrangements occur in this region in the crystal structure which could provide more pronounced shifts (Kim et al., 1974; Robertus et al., 1974). The assignments as shown in Table I were made to minimize the discrepancies and to be consistent with the melting behavior in which, at 60 °C (Figure 4, spectrum B),

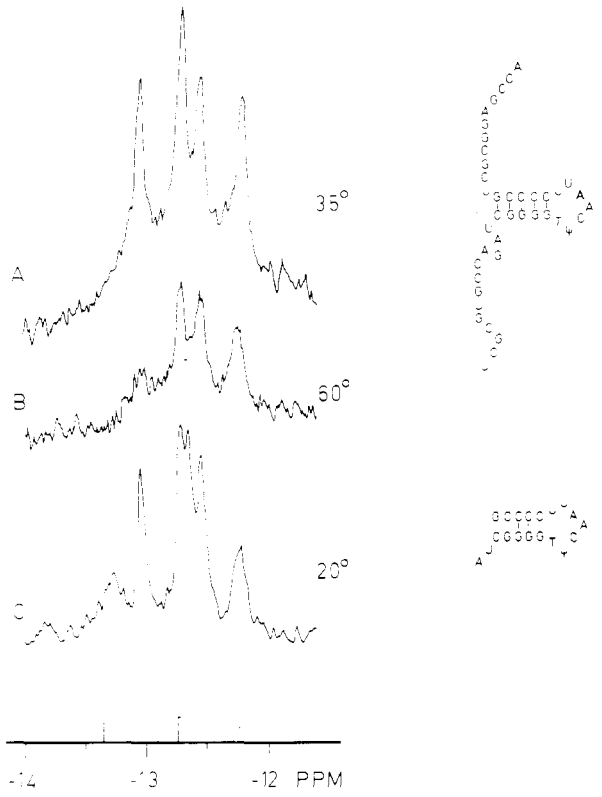

FIGURE 4: Proton NMR spectra (270 MHz) of the CCA half-molecule at 35 °C (spectrum A) and 60 °C (spectrum B) and the TψC hairpin at 20 °C (spectrum C). All samples were prepared in the same buffer listed in the Materials and Methods section. The fragment concentration was approximately 1 mM. The positions marked at the bottom of the figure are those calculated from the ring current calculations for the base pairs indicated in the figure.

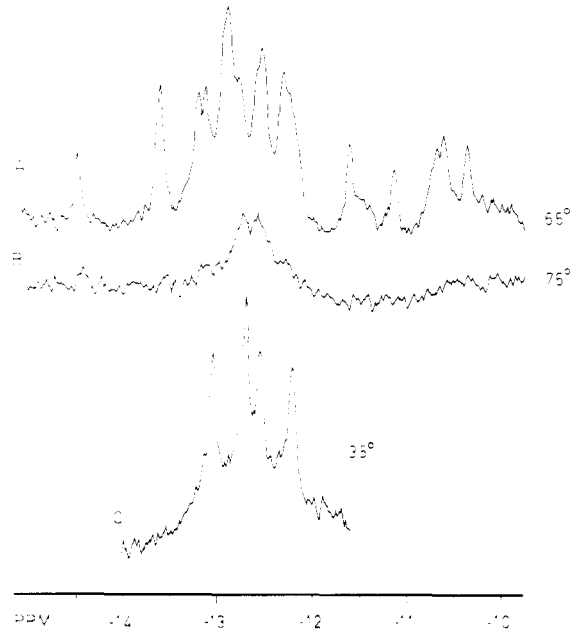

FIGURE 5: A comparison of the 270-MHz proton NMR spectra of the intact $tRNA^{Asp}$ at 65 and 75 °C with the spectrum of the CCA half-molecule at 35 °C.

the resonance at −13.0 ppm broadens and disappears first. This suggests that the −13.0-ppm peak should be assigned to G·C-48 since the helix probably unwinds from the open end of the hairpin. Since the CCA half-molecule could combine to form other base pair combinations in addition to those of the TψC helix we compared these spectra with the spectrum of the isolated TψC hairpin (Figure 4C). The distribution of resonances and their positions are essentially the same as those of the CCA half-molecule, indicating that the spectra of both fragments are representative of the five G·C base pair hydrogen-bonded proton resonances of the TψC arm. In addition to the five resonances assigned to the TψC stem base pair protons there is also a partial resonance at −13.3 ppm in spectrum C which has no analogue in the fragment spectra A and B. This resonance originates from a bimolecular association of the hairpin fragment which manifests itself in three additional resonances below −13 ppm at temperatures between 0 and 20 °C. These additional resonances melt out, however, by 25 °C leaving only the five resonances representative of the TψC stem which do not melt out until much higher temperatures (C. W. Hilbers and G. T. Robillard, manuscript in preparation). A biphasic melting has also been observed by Coutts et al. (1974) in their optical melting experiments on the same fragment.

Assignment of the Resonances in the Native Molecule. Having observed the resonances from the TψC stem base pairs in the fragment preparation it is possible to compare the fragment spectra with the high-temperature spectra of the native molecule where only the TψC stem should be intact. In Figure 5 it is evident that the resonances of the TψC stem fragment can also be observed in the whole molecule spectra at 75 °C even though the resonance line widths in spectrum B are exchange broadened as a result of the limited lifetime of this helix. In the 65 °C spectrum all resonances are narrow but resonances from regions other than the TψC stem are also present. We conclude, therefore, that the positions of the five base pair proton resonances of the TψC stem are essentially the same in the native molecule as in the fragment.

Using the assignments already obtained for the intact molecule TψC arm by way of the fragment spectra, we have assigned the rest of the resonances below −11.5 ppm on the basis of resonance positions predicted from ring current shift calculations and simulation of spectra. Two assumptions were made in these assignments: (i) in the 65 °C spectrum the TψC arm is completely intact and contributes five proton resonances, each with unit intensity, at the positions assigned from the fragment spectra; (ii) the anticodon arm and the acceptor stem are melting together in the vicinity of 65 °C since there is a widespread decrease in the intensity between 50 and 70 °C (Figure 3) and these resonances are estimated to have an intensity of 0.7 proton each, at 65 °C, compared with unity for the resonances of the TψC arm. The dotted line in Figure 6A is the computer simulation of the 65 °C spectrum using the resonance positions marked at the bottom of the figure and the intensities stated above. Since the simulated spectrum fits the observed 65 °C spectrum so closely the consistency of our assumptions concerning the resonance intensities in this spectrum was checked by also simulating the 50 °C spectrum. We have previously stated that, at 50 °C, all three helices are completely intact and the three resonances tentatively assigned to tertiary structure are also present. Therefore, the 50 °C spectrum was simulated using the same area for a one-proton peak as in the 65 °C spectrum simulation, but all proton resonances were brought to full intensity and the three resonances which melt early were also added. The only adjustment was that the resonances for A·U-1 and A·U-7 were set at 0.8 proton. With this single adjustment there is nearly a

perfect computer fit to the observed spectrum. The extra intensity at 13.3 ppm could not be simulated by an additional proton and, furthermore, was not present in the 360-MHz spectrum of another sample of the same material.

Table I presents the calculated resonance positions for the proposed helical regions of yeast tRNA^{Asp} and compares these predicted positions with those assigned on the basis of the fragment studies and the computer simulations. There are only three resonances for which there is a large discrepancy between the observed and calculated positions. All three are at the ends of helical regions. G·C-52 has already been mentioned. G·C-27, at the top of the anticodon arm, should receive some shift contribution from A-44 and G-26; however, they may not be completely stacked and, as we have shown in Table I, G·C-27 can take a position between −13.0 and 11.8 ppm depending on the contribution from A-44 and G-26. This same discrepancy has been found for *E. coli* tRNA^{Glu} for the resonance at the top of the anticodon helix (Hilbers and Shulman, 1974). Base pair A·U-1 also appears to be abnormally shifted. It is 0.36 ppm to higher field than calculated when the contribution of G-72 is fully included. It is possible that G-72, which is not hydrogen bonded, may not be stacked in the normal helical manner and thereby causes a larger than predicted shift. The root-mean-square error between the observed resonance positions and those calculated for each base pair of the 14 resonances without G·C-27 is $(\Delta^2/14)^{1/2} = 0.19$ ppm, while the average deviation is 0.01 ppm. If we were to neglect the other three end resonances, A·U-1, G·C-31, and G·C-52, adjacent to nonhelical structures the root-mean-square error would be 0.10 ppm.

G·U Base Pairs. In Figure 2 the region from −11.4 to −10 ppm contains a considerable amount of resonance intensity: at 35 °C it corresponds to about 12 protons. Slightly more than half of this has disappeared, however, by 60 °C (Figure 3) in the same temperature interval during which the three distinct resonances assigned earlier to tertiary structure hydrogen bonds also disappear. At that point just four resolved resonances are left in this region in the 65 and 70 °C spectra. These individual resonances have the same integrated intensity as the resolved resonances assigned to A·U-7 and G·C-6 at −14.5 and −11.65 ppm, respectively. Furthermore, their intensities have the same temperature dependence as A·U-7 and G·C-6, decreasing with temperature without changing their widths. Since resonances above −11.4 ppm are at too high a field position to be normal Watson-Crick hydrogen-bonded protons, we suggest that they are ring NH protons in slow exchange with solvent protons. The acceptor and anticodon helices each contain one G·U base pair (G·U-5 and G·U-30) which cannot form the normal Watson-Crick hydrogen bond since both ring nitrogens are protonated. Thus, two proton resonances should come from each G·U pair. The positions of these resonances in pure H_2O have been determined by proton NMR measurements in mixtures of Me_2SO and H_2O at various ratios and extrapolating the chemical shifts to 100% H_2O. These positions are −11.25 for the G-NH and −11.4 ppm for the U-NH (Y. Wong, Ph.D. Dissertation). The resonances we observe are shifted slightly upfield from these positions presumably because of ring current shifts from neighboring bases.

The DHU Arm. The present NMR study of yeast tRNA^{Asp} shows no indication of Watson-Crick base pairing in the dihydrouridine arm of the intact tRNA molecule. This is in contrast to previous studies on several tRNAs

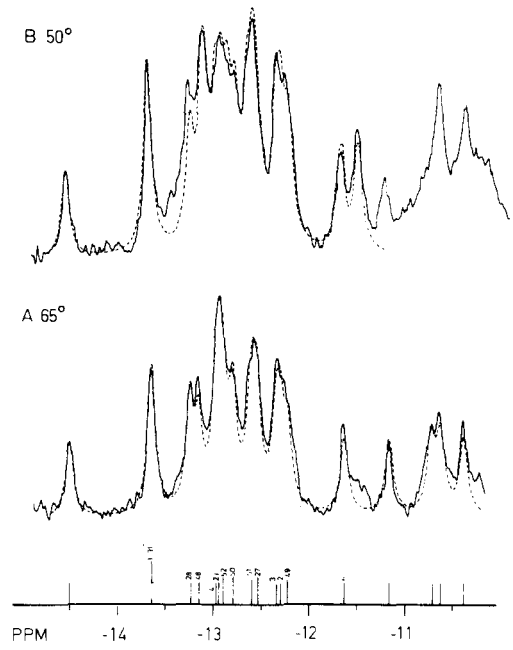

FIGURE 6: A comparison of the observed (solid line) and computer simulated spectra (dotted line) at 50 and 65 °C. The computer simulations were done as described in the Materials and Methods section. The positions used for the computer simulation are marked at the bottom of the figure. They are the same as those marked "observed positions" in Table I. The intensity input has been described in the text.

where resonances from base pairs of the dihydrouridine arm were identified. There are two possible reasons for not seeing these resonances. Either the hydrogen bonds exist but the lifetimes of the protons in the helix are too short to be observable (i.e., <5 ms) or the Watson-Crick hydrogen bonds and the corresponding helical structure do not exist in this molecule. We shall show below that a consistent interpretation of the spectra is only obtained if the bases of the dihydrouridine arm are not paired. This is best discussed after we examine, in the next section, the −10- to −11.5-ppm region of the NMR spectra.

At low temperature, 35 °C, there is a large intensity in the −10- to −11-ppm region in the NMR spectrum of tRNA^{Asp} from yeast. In addition to the evidence presented above for assigning some of these resonances to non-hydrogen-bonded G and U ring nitrogen protons, we have presented separately (Reid et al., 1975) a comparison of yeast tRNA^{Asp} with three *E. coli* tRNAs each of which contain only one G·U base pair in their cloverleafs and the intensity ratios in the −10- to −11.3-ppm region are as expected from the present assignments. Additional correlations of this sort are available from published work. Both yeast tRNA^{Phe} and *E. coli* tRNA^{fMet} have one G·U base pair in their cloverleafs and intensities of only three or four protons in this region. In the present spectra of yeast tRNA^{Asp} the −10- to −11.3-ppm region has an integrated intensity of about 12 protons at the lowest temperature measured of 35 °C. This intensity was determined by its integrated area relative to that below −11.3 ppm which was assumed to correspond to 20 protons for the reasons discussed above. These 12 protons include four from G·U-5 and -30 as seen in the 70 °C spectrum (Figure 3). The remaining eight protons are lost when the temperature is raised to 55 °C. Since the four protons already assigned to the G and U protons of the G·U pairs in the acceptor stem and anticodon arm fall in this region we suggest that four additional protons are

reasonably assigned to G·U-10 and G·ψ-13 and that two more could come from non-hydrogen-bonded U-11 and U-12. Only two protons are left unaccounted for. If the four from G·U-10 and G·ψ-13 have long lifetimes (>5 ms) and are in slow exchange with solvent, it would be inconsistent to argue that the hydrogen-bonded protons of A·U-11 and -12, located between G·U-10 and G·ψ-13, exchange rapidly with water and are not observed below −14 ppm. Hence, we conclude that the self-consistent interpretation of these data is that A·U-11 and A·U-12 are not hydrogen bonded and that the dihydrouridine arm does not have a normal base paired conformation.

Comparison with Temperature-Jump Results. Coutts et al. (1974) have reported on the optical and temperature jump studies of yeast $tRNA^{Asp}$ melting. In 0.5 M NaCl (no Mg^{2+}) a melting transition was observed, peaked around 55 °C. This differential melting curve could be resolved in a temperature-jump experiment into three melting transitions peaked around 51, 55, and 60 °C, which were designated transitions I, II, and III, respectively. Transition III was ascribed to the perturbation of the single-strand stacking equilibrium and will not be detectable in the present NMR experiments.

Transition I at 51 °C consists of a very slow process (~200 ms) and, as has been explained (Hilbers et al., 1976), such a melting process will manifest itself in the NMR spectrum by a shrinking of resonances. Such a shrinking is observed indeed for the resonances of the acceptor and anticodon stems which melt simultaneously. Apparently this melting is decoupled under the conditions of the temperature-jump experiments since transition I is assigned to the melting of the tertiary structure, the DHU arm, and the acceptor arm, while no analogue of transition II is observed in the NMR experiments. The possible candidate for this transition would be the TψC arm. It has a melting temperature >90 °C in 0.5 M Na^+ solution and, in agreement with this, in the NMR experiment the lifetime of its double helical stem is still >5 ms at 75 °C.

Returning to transition I, the NMR results indicate that the DHU arm is not formed at 51 °C in the presence of Mg^{2+} and based on this evidence we do not expect it to contribute to transition I observed in 0.5 M Na^+ (no Mg^{2+}).

The ring current shift predictions indicate that, if both A·U-7 and A·U-11 were intact, two resonances should occur below −14 ppm. Since only one resonance is present and it disappears simultaneously with the rest of the resonances of the acceptor stem it has been assigned to A·U-7 and not to A·U-11. Possible candidates for the A·U resonances of the DHU arm would be two of the three resonances at −13.08, −12.64, and −11.45 ppm, which we lose below 50 °C. These resonances are too far upfield, however, to be attributed to the base pair hydrogen-bonded protons of the normal Watson-Crick base pairs of the dihydrouridine arm. Since we are able to account for all other base pair protons in the cloverleaf by the remaining resonances, these must be assigned to tertiary structure hydrogen-bonded protons.

Tertiary Structure Hydrogen Bonds. At 35 °C the resonances assigned to tertiary structure hydrogen bonds are at −13.05, −12.64, and −11.45 ppm. Examination of the crystal structure of yeast $tRNA^{Phe}$ suggests that the tertiary structure Watson-Crick pair of G-19–C-56 should have a resonance position near −13.0 ppm, while the hydrogen bond of G-46 to N_7 of G-22 should be shifted toward −12.5 ppm. This agrees rather well with the resonances observed at −13.08 and −12.64 ppm and might be the correct assignment. These possibilities, as well as an assignment of the −11.45-ppm resonance, are discussed elsewhere (Reid et al., 1975) and are being investigated more fully at the present time.

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