Chemically Induced Dynamic Nuclear Polarization Studies of Yeast tRNA^{Phe} †

Elizabeth F. McCord, Kathleen M. Morden, Ignacio Tinoco, Jr., and Steven G. Boxer*

ABSTRACT: Chemically induced dynamic nuclear polarization (CIDNP) has been observed from yeast tRNA^{Phe} following reaction with photoexcited riboflavin. At 20 °C, several resonances of tRNA in the native form show polarization; previous work predicts that only guanosine and its derivatives in single-stranded regions are likely to become polarized [McCord, E. F., Morden, K. M., Pardi, A., Tinoco, I., Jr., & Boxer, S. G. (1984) *Biochemistry* (preceding paper in this issue)]. The methyl protons of m_2^2G-26 show strong negative spin polarization, indicating that this residue is accessible. The solvent accessibility of this residue has not been previously demonstrated. In addition, two positively polarized aromatic resonances are observed, which are likely due to two or more G(H8) protons, including those of G-20, m_2^2G-26, and/or Gm-34. For temperatures below 50 °C, a negatively polarized signal in the aromatic region is shown to arise from cross relaxation with the methyl group protons of m_2^2G-26. This indicates the proximity of an aromatic proton, probably H2 of A-44, to the methyl groups of m_2^2G-26. At higher temperatures, the CIDNP spectra show polarization of several additional G resonances, including those of m^2G-10. These changes in the CIDNP spectra reflect melting of the tertiary and secondary structure of the tRNA. This work is the first use of CIDNP to study a large nucleic acid molecule and exemplifies the value of this technique in probing single-stranded and solvent-accessible regions of tRNA.

There has been a great deal of interest in the conformation of tRNA in solution and in the effects of various perturbations such as aminoacylation, pH, Mg^{2+} concentration, and anticodon–codon base pairing on this conformation. Many methods, including nuclear magnetic resonance (NMR),[1] circular dichroism , and chemical modification, have been used to study the conformation of yeast tRNA^{Phe} in solution (see Figure 1 for the secondary structure of tRNA^{Phe}). The results of these studies generally agree with those based on the yeast tRNA^{Phe} crystal structure (Holbrook & Kim, 1983).

The NMR method chemically induced dynamic nuclear polarization (CIDNP) is well suited to probing macromolecular surface topology in solution. CIDNP is observed as enhanced absorption or emission of the NMR resonances of the products of radical pair reactions. Kaptein and co-workers have observed CIDNP of the purine bases guanine and adenine following their reversible reaction with photoexcited flavins (Kaptein et al., 1979). G(H8) shows positive polarization, and A(H8) shows positive polarization at low phosphate concentrations but negative polarization at high phosphate concentrations (Scheek et al., 1981). In the previous paper (McCord et al., 1984) on CIDNP from nucleotides and oligonucleotides, we found that G shows CIDNP when it is in single-stranded regions and accessible to the photoexcited flavin; A shows polarization only in the absence of accessible G's. Use of the dye 8-bromo-8-desmethylriboflavin (8BrF) increases the G polarization by a factor of 2 and reverses the sign of the A(H8) polarization at high phosphate concentrations. The sign of the A(H8) polarization becomes negative at high phosphate concentrations when riboflavin is used as the dye. Thus, varying the dye and phosphate concentration provides a convenient method for distinguishing the H8 NMR resonances of A and G bases when they become spin polarized. The reaction with photoexcited riboflavin can also induce polarization of the modified or rare bases m^1G, m_2^2G, m^2G, Gm, m^7G, and Ψ.

In this paper, we report CIDNP studies of yeast tRNA^{Phe}. CIDNP has been used extensively by Kaptein and co-workers to study the surface accessibility of His, Trp, and Tyr residues in proteins to photoexcited flavins. We have used CIDNP in a similar fashion to determine the accessibility of G residues in tRNA^{Phe}. Because CIDNP requires the target residue to be accessible to the photoexcited dye, polarization is expected only from single-stranded, solvent-accessible regions of oligomers. The observed CIDNP in tRNA is the result of a nondestructive, cyclic electron-transfer reaction; the CIDNP reflects the accessibility of the G bases in the native (nonmodified) tRNA molecule.

At 20 °C, both aromatic and aliphatic resonances of yeast tRNA^{Phe} show polarization. The variations in this polarization as a function of temperature, phosphate concentration, dye, and excision of the Y base have been studied. Two of the polarized aliphatic resonances are assigned to the methyl groups of m_2^2G-26 and m^2G-10; assignments of the polarized aromatic resonances are suggested on the basis of chemical-modification studies (Rhodes, 1975), NOE results, and observed CIDNP dipolar cross relaxation between resonances.

Experimental Procedures

7-Methyl-8-bromo-10-(1-D-ribityl)isoalloxazine (denoted 8BrF) was a generous gift from Dr. J. Lambooy at the University of Maryland. The 8BrF used in the tRNA experiments contained approximately 20% of the corresponding chloro compound (8ClF). Yeast tRNA^{Phe} was purchased from

† From the Department of Chemistry, Stanford University, Stanford, California 94305 (E.F.M. and S.G.B.), and the Department of Chemistry and Laboratory of Chemical Biodynamics, University of California, Berkeley, California 94720 (K.M.M. and I.T.). *Received July 19, 1983.* This work was supported by National Institutes of Health Grants GM 27738 (S.G.B.) and GM 10840 (I.T.) and by the Department of Energy, Office of Energy Research, under Contract 03-82ER 60090.000 (I.T.). The 360-MHz NMR spectra were obtained at the Stanford Magnetic Resonance Laboratory supported by National Science Foundation and National Institutes of Health Grants GR 23633 and RR 0711, respectively. E.F.M. is an NSF Pre-doctoral Fellow, K.M.M. was supported by NIEHS Training Grant ES 07075, and S.G.B. is an Alfred P. Sloan and Camille and Henry Dreyfus Teacher-Scholar Fellow.

[1] Abbreviations: CIDNP, chemically induced dynamic nuclear polarization; 8BrF, 7-methyl-8-bromo-10-(1-D-ribityl)isoalloxazine; 8ClF, 7-methyl-8-chloro-10-(1-D-ribityl)isoalloxazine; EDTA, ethylenediaminetetraacetic acid; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement. Standard abbreviations are used for nucleic acids (Davies, 1978; Nishimura, 1978).

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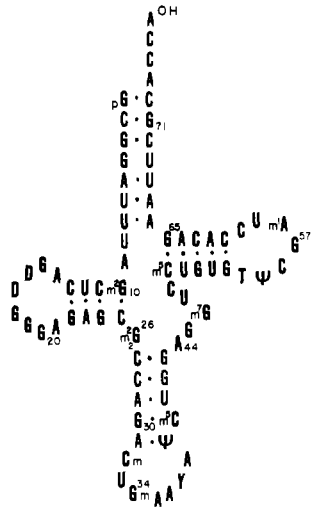

FIGURE 1: Secondary structure of yeast $tRNA^{Phe}$.

Boehringer-Mannheim. The tRNA samples were prepared by repeated dialysis against buffer containing 0.15 M NaCl, 5 mM phosphate, and 1 mM EDTA at pH 7 and then dialyzed repeatedly against the same buffer without EDTA, lyophilized, and dissolved in D_2O to give a final concentration of ~1.5 mM. This sample was then added to the lyophilized dye. The Y base was excised from $tRNA^{Phe}$ (the resulting molecule will be referred to as $tRNA^{Phe}$-Y) by dilution of the $tRNA^{Phe}$ sample (containing dye) to 2.5 mL, adjustment of the pH to 2.9 with 1 N HCl, heating for 4 h in the dark at 37 °C, neutralization to pH 7, lyophilization, repeated dialysis against buffer containing 5 mM phosphate and 0.15 M NaCl, pH 7, followed by addition of 8BrF, lyophilization, and dissolution in D_2O (Thiebe & Zachau, 1968). The pH of deuterated buffers refers to the uncorrected pH meter reading.

NMR data were obtained on the 360-MHz NMR spectrometer at the Stanford Magnetic Resonance Laboratory. The chemical shifts are referenced to internal 3-(trimethylsilyl)-1-propanesulfonic acid (DSS); DSS was added to the tRNA samples following the CIDNP experiments. Presaturation was used to suppress the residual HDO resonance. The temperature was 20 ± 1 °C unless otherwise indicated. The photo-CIDNP apparatus and data collection system have been previously described (McCord et al., 1981). CIDNP spectra were obtained with a 90° radio frequency pulse. Enhancement factors are defined as the difference between the integrated intensity of a polarized peak and its unenhanced dark intensity, divided by the latter. These values can be interpreted relative to each other, but not in absolute terms, as this depends on sample geometry, dye concentration, etc.

Results

Aromatic Region. Two positively polarized peaks are observed in the aromatic region of the CIDNP spectra of $tRNA^{Phe}$ below 60 °C. The spectrum at 20 °C is shown in Figure 2. A negatively polarized peak is also observed in the aromatic region at temperatures below 50 °C. Addition of 0.15 M phosphate or use of the dye 8BrF did not change the sign or the pattern of polarization. The negatively polarized peak at 7.7 ppm disappeared when the intense, negatively polarized resonance at 2.5 ppm was irradiated with radio frequency (rf) during the light pulse (0.5-s duration) (Closs & Czeropski, 1977). There was no change in the CIDNP pattern or intensity when any of the other polarized resonances was irradiated with rf during the light pulse. As the temperature was increased from 60 to 85 °C, the number and intensity of polarized peaks increased dramatically (Figure 3).

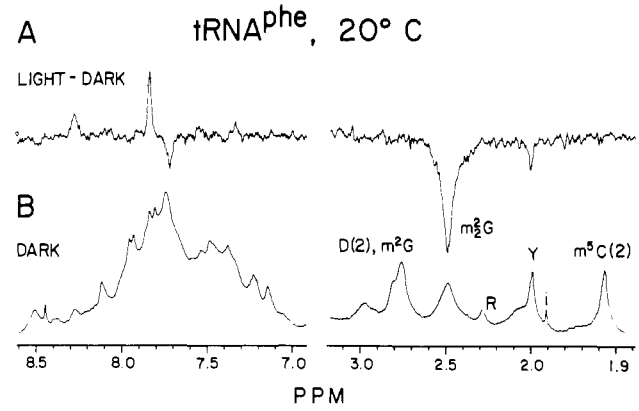

FIGURE 2: ^1H NMR spectra (360 MHz) of yeast $tRNA^{Phe}$ (1.5 mM) in pH 7 buffer containing 0.15 M NaCl, 5 mM phosphate, and 0.4 mM 8BrF at 20 °C. i indicates an impurity in the buffer. R indicates the methyl resonance of the dye. (A) Light minus dark difference spectrum (one accumulation); (B) dark spectrum (500 accumulations).

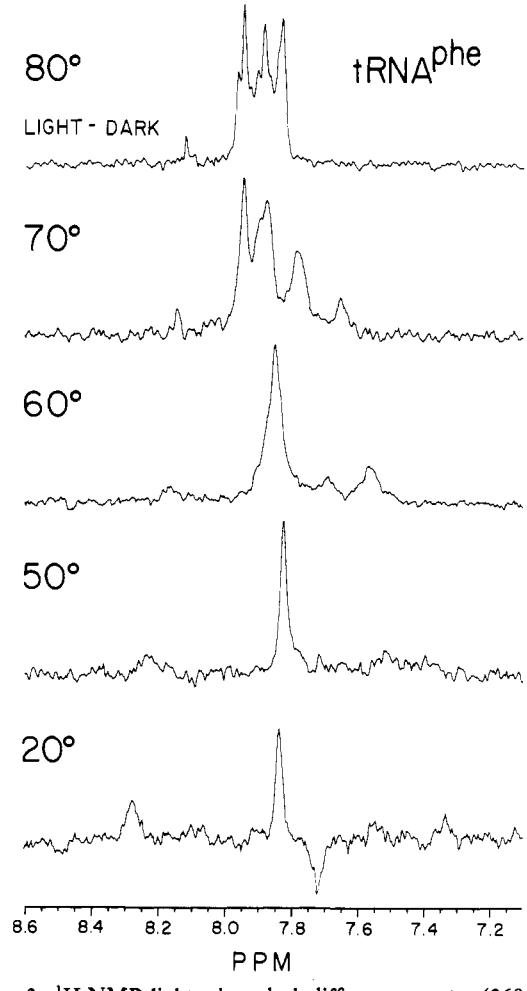

FIGURE 3: ^1H NMR light minus dark difference spectra (360 MHz, one accumulation each) of yeast $tRNA^{Phe}$ (~1.5 mM) in pH 7 buffer containing 0.15 M NaCl, 5 mM phosphate, and 0.4 mM 8BrF at 20, 50, 60, 70, and 80 °C.

The negatively polarized peak at 7.7 ppm was not observed at 50 °C and above. The chemical shifts and relative intensities of the polarized peaks as a function of temperature did not change substantially when the Y base was excised, although the line width of the major polarized signal was somewhat greater for $tRNA^{Phe}$-Y between 40 and 60 °C than the line width of the corresponding polarized signal for $tRNA^{Phe}$.

Aliphatic Region. Two negatively polarized resonances are present in the aliphatic region of the $tRNA^{Phe}$ CIDNP spec-

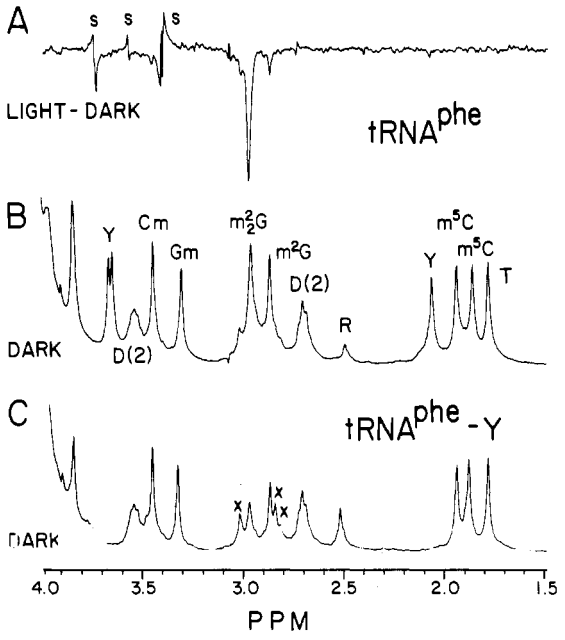

FIGURE 4: ^{1}H NMR spectra (360 MHz) at 85 °C. (A) Light minus dark difference spectrum (one accumulation) of yeast tRNA^{Phe} (~1.5 mM) in pH 7 buffer containing 0.15 M NaCl, 5 mM phosphate, and 0.4 mM 8BrF. (B) Dark spectrum of yeast tRNA^{Phe} (~1.5 mM) in pH 7 buffer containing 0.15 M NaCl, 5 mM phosphate, and 0.4 mM 8BrF (252 accumulations). s indicates spikes arising from the rf used to saturate the solvent. These have random phase and thus are not present in spectra consisting of many accumulations. R indicates the methyl resonance of the dye. (C) Dark spectrum of yeast tRNA^{Phe}-Y (~1.5 mM) in pH 7 buffer containing 0.15 M NaCl, 5 mM phosphate, and 0.4 mM 8BrF (252 accumulations) showing the complete absence of the Y base. x indicates resonances that may be due to photochemical degradation (see text).

trum (Figure 2). Both negative peaks are also observed upon addition of 0.15 M phosphate, when the dye 8BrF was used rather than riboflavin, and in tRNA^{Phe}-Y, though the relative intensities of the two peaks varies somewhat from sample to sample and as a function of temperature, phosphate concentration, and dye used. The polarized peak at 2.0 ppm is not observed above 50 °C. Above 60 °C an additional negatively polarized resonance is observed (3.3 ppm at 60 °C). The methyl and methylene resonances of tRNA^{Phe} are assigned on the basis of the chemical shifts, integrals, and splittings of the signals and agree with those of Davanloo et al. (1979). Minor differences in the chemical shifts between our spectra and theirs are most likely due to differences in concentration, buffer composition, and magnesium concentration. The positions of the m$_2^2$G-26 methyl resonances are especially easy to determine by inspection of the corresponding CIDNP spectra. The Y base assignments are definitive on the basis of the absence of these resonances in the NMR spectrum of tRNA^{Phe}-Y (Figure 4C), which is otherwise very similar to the spectrum of tRNA^{Phe} at all temperatures (Davanloo et al., 1979).

The aliphatic polarization of the tRNA^{Phe}-Y sample decreases at high temperature even though the aromatic polarization remains strong. There are several unassigned peaks between 2.9 and 3.3 ppm in the tRNA^{Phe}-Y dark NMR spectrum at 85 °C. These could be due to sample heterogeneity, chemical degradation of the molecule, or photochemical degradation of selected bases (m$_2^2$G-26 and m^{2}G-10). We favor the latter explanation, as this sample had been used repeatedly in CIDNP experiments, similar peaks of much smaller intensity are seen in the tRNA^{Phe} spectrum at 85 °C (this sample had been subjected to far less laser irradiation), and degradation of other molecules (e.g., ApGpCpU) has been observed after many CIDNP experiments. It has been reported that chemical degradation of tRNA^{Phe} occurs in the presence of magnesium at high temperatures. Our samples probably contain ~5 mM Mg^{2+} (vide infra), so chemical degradation is also a possibility. Photochemical degradation could well be responsible for the loss of aliphatic polarization in tRNA^{Phe}-Y at high temperatures. Experiments were done from low to high temperatures; if the temperature was subsequently decreased, the pattern of polarization was as expected, but the intensity was greatly reduced. Samples that had been used at greater than 40 °C were not used in subsequent experiments at lower temperatures. The NMR spectrum of tRNA^{Phe} is unaffected by the addition of dye, although the chemical shifts and line widths of some of the flavin resonances are somewhat different when tRNA^{Phe} is present.

Discussion

Heretofore, CIDNP has only been detected in very small oligonucleotides; a key result of this work is the observation of CIDNP in the much larger tRNA molecule. On the basis of our previous work (McCord et al., 1984), we expect that only the accessible G bases in the single-stranded loop regions of the tRNA molecule will show polarization. These regions of the tRNA structure are thought to be involved in critical contacts with other components in protein synthesis. The NMR features associated with these residues, especially in the aromatic region, are very complex and poorly understood in contrast to the wealth of data that is available for the imino protons in double-stranded regions (Hurd & Reid, 1979; Johnston & Redfield, 1981). Thus, the CIDNP method offers complementary information.

Examination of a model based on the crystal structure of yeast tRNA^{Phe} (see also Figure 1) suggests that three G's (G-57, G-20, and Gm-34) are in loop regions and are not involved in tertiary hydrogen-bonding interactions with other bases (Holbrook et al., 1978). G-57 is in the interior of the TΨC loop, whereas G-20 and Gm-34 are clearly located on the outside of the molecule and are readily accessible to the solvent. Chemical-modification studies with *N*-cyclohexyl-*N'*-[β-(4-methylmorpholinium)ethyl]carbodiimide-*p*-toluenesulfonate at 37 °C (Rhodes, 1975) and kethoxal at 25 °C (Litt, 1971) have indicated that G-20 and Gm-34 are accessible (G-18 is slightly accessible to the carbodiimide reagent). Chemical-modification studies at 37 °C with the reagent dimethyl sulfate, which reacts at G(N7), indicate that G-18, G-19, G-30, G-45, G-71, and Gm-34 are fully accessible, while G-1 and G-65 are partially accessible (Peattie & Gilbert, 1980). Measurements of the rates of tritium exchange into the H8 positions of the purines at 37 °C indicate that Gm-34 and G-18 are probably the most solvent accessible G's and m$_2^2$G-26 is among the six or seven most accessible G's (the accessibility of G-20 could not be determined in these experiments) (Gamble et al., 1976). Chemical-modification with the carbodiimide reagent has also been studied as a function of temperature up to 55 °C (Rhodes, 1977). G-4, G-15, G-18, G-20, Gm-34, G-45, m^{7}G-46, and also possibly m^{2}G-10, G-22, and G-65 were found to be accessible in the early stages of thermal unfolding. There was no evidence for modification of m$_2^2$G-26 up to 55 °C.

Because m$_2^2$G-26 is found to exhibit strong spin polarization in contrast to these chemical-modification studies, we consider its chemistry in greater detail. m$_2^2$G-26 is thought to be H-bonded to A-44 through one or two hydrogen bonds, although the two purines are twisted in a propellor fashion relative to each other (Holbrook et al., 1978). The dimethylamino group is oriented directly outward from the molecule into solution.

m_2^2G-26 has not been affected in the chemical-modification studies, but the appropriate atoms necessary for reaction may not be accessible to these reagents, and it is possible that even a fully accessible m_2^2G would not react with these reagents. The kethoxal adduct involves dehydration with one of the amino hydrogens and may not be stable when these are not available. The carbodiimide reagent is presumed to react at N1; the dimethylamino group may interfere sterically with this reaction.

By contrast, CIDNP is generated by reversible one-electron transfer from G or its derivatives to the photoexcited flavin (McCord et al., 1984). m_2^2G is nearly insoluble at 25 °C and shows very weak negative polarization of the methyl protons. The solubility of this compound is very temperature dependent, however, and above 40 °C strong CIDNP was observed for H8 and for the methyl protons. At 40 °C, the ratio of the enhancements of the H8 to the methyl resonance is 1 to −2.5. Separately, m_2^2G and G have almost the same enhancment factor for the H8 proton when each is 0.3 mM (0.25 mM riboflavin, pH 7, 66 mM phosphate, 40 °C). In an equimolar mixture of these two compounds under the same conditions (except that the bases were each 1 mM), the H8 again showed almost identical enhancements. m^2G is also increasingly soluble at higher temperatures. It, too, shows positive H8 polarization and negative methyl polarization.

At 20 °C and above, it is evident from Figures 2A and 4A that the m_2^2G-26 methyl protons show strong CIDNP. The CIDNP in the aromatic region indicates that at least two G's (presumably Gm-34, G-20, and/or m_2^2G-26) have spin-polarized H8 resonances and therefore react with photoexcited riboflavin at 20 °C. Our studies of smaller oligomers (McCord et al., 1984) show that accessible A residues do not exhibit spin polarization in the presence of accessible G residues. Thus we do not believe that the polarization in the aromatic region is due to A in spite of the fact that the amino groups of several A residues are certainly accessible. This is confirmed by the observation that the sign of the polarization in the aromatic region is unaffected by high phosphate concentrations, in contrast with what has been shown for A itself (Scheek et al., 1981).

We have shown that the negatively polarized resonance at 7.7 ppm is spin polarized through cross relaxation from the m_2^2G-26 methyl protons. The fact that the transferred polarization is of the same sign is expected given the long correlation time of the tRNA molecule (Kaptein & Edzes, 1979). This polarized aromatic resonance must be from a nonexchangeable proton in close proximity to the m_2^2G-26 methyl groups; examination of the crystal structure suggests that H6 of C-27, H8 of m^2G-10, or H2 of A-44 are within 6 Å of the m_2^2G-26 methyl group. A nuclear Overhauser enchancement (NOE) has been reported from the methyl group of m_2^2G-26 to an aromatic proton at 8.4 ppm and was assigned to m^2G-10(H8) in a sample of yeast $tRNA^{Phe}$ (0.1 M NaCl, 10 mM phosphate, pH 7, 95% H_2O, no added Mg^{2+}) (Sanchez et al., 1980); several other NOE's to the aromatic region were observed but were not assigned. D. Hare and B. Reid (personal communication) have observed NOE's at 32 °C from the m_2^2G-26 methyl group to three resonances due to nonexchangeable protons at 8.41, 7.94, and 7.71 ppm vs. DSS [10 mg of yeast $tRNA^{Phe}$ (Boehringer-Mannheim) in 0.5 mL of deuterated pH 7, 10 mM phosphate buffer; Mg^{2+} concentration estimated to be ~5 mM]. The intensities of the NOE's increased from low to high field. On the basis of the work of Sanchez et al. (1980) and of the distances of protons from the m_2^2G-26 methyl groups in the crystal structure, we suggest that the 8.41 ppm resonance is m^2G-10(H8), the 7.94 ppm resonance is C-27(H6), and the 7.71 ppm resonance is A-44(H2). The strongest Overhauser-enhanced resonance at 7.71 ppm corresponds nicely to the cross-polarized resonance we observe at 7.7 ppm in the CIDNP spectrum, suggesting that this is A-44(H2). This cross polarization is no longer observed at temperatures above 50 °C, presumably due to local melting of the structure.

The origin of the polarization at 2 ppm (20 °C) is not known. The fact that it is still present in the CIDNP spectrum of $tRNA^{Phe}$-Y rules out the possibility that it is from the Y base, in spite of the fact that it occurs at nearly the same chemical shift. The line width of this polarized peak is considerably narrower than those of other peaks, so it is likely to be a minor impurity in the solvent.

The temperature dependence of the CIDNP spectrum is quite striking: several additional polarized resonances are observed starting at about 60–65 °C (Figure 3). Weak polarization of the m^2G-10 methyl protons is also observed beginning at this temperature (data not shown). It is thought that the structure of $tRNA^{Phe}$ approximates the native, active form in the presence of high NaCl concentrations whether Mg^{2+} is present or not (Rhodes, 1977). In the presence of Mg^{2+}, the tertiary structure of $tRNA^{Phe}$ is stable up to approximately 60 °C, where it melts cooperatively. The melt starts at lower temperatures in a sequence of several steps in the absence of Mg^{2+}, but the secondary structure stays relatively intact until greater than 40 °C (Johnston & Redfield, 1981). Comparison of our dark NMR spectra as a function of temperature with those in the literature (Davanloo et al., 1979; Robillard et al., 1977) suggests that our samples contain between 0 and 10 mM Mg^{2+}. Upon melting of the tertiary structure, we would expect G-15, G-18, G-19, G-45, G-46, and G-57 to become more accessible. Most of these, as well as G-4, m^2G-10, G-22, and G-65, become accessible to chemical modification at higher temperatures (Rhodes, 1977). Consistent with this, we observed several additional polarized aromatic resonances beginning at ~60 °C (Figure 3).

To date, there has been relatively little progress in analyzing the aromatic regions of tRNA NMR spectra (Schmidt & Edelheit, 1981; Johnston & Redfield, 1981), in contrast to the wealth of information available from the slowly exchanging imino protons in double-stranded regions (Hurd & Reid, 1979; Johnston & Redfield, 1981). It is noteworthy that CIDNP selectively detects the single-stranded regions. These are mechanistically of great importance and have thus far not been analyzed in detail by NMR. CIDNP can also monitor the accessiblity of many of the modified bases, which may be important in determing protein–tRNA and ribosome–tRNA binding sites. This example demonstrates that higher oligomers of nucleic acids are definitely amenable to study with CIDNP and opens the possibility of much wider studies.

Acknowledgments

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Registry No. 8BrF, 40371-66-2; guanosine, 118-00-3.

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Neutron Small-Angle Scattering Studies of Ribonuclease in Mixed Aqueous Solutions and Determination of the Preferentially Bound Water†

M. S. Lehmann* and G. Zaccai

ABSTRACT: Neutron small-angle measurements of ribonuclease A in mixed solutions of ethanol–water and glycerol–water have been used to estimate the region near the molecule that only contains water. When glycerol is used as probe, the region corresponds to an interaction parameter ξ of 0.23 ± 0.05 g of water/g of protein. For ethanol ξ is −0.07 ± 0.05, corresponding to the macromolecule being equally accessible to ethanol and water. The observations of the radius of gyration in the mixed solutions are used to show that the volume excluding glycerol is found on the exterior of the protein.

The biological activity of soluble proteins is most often studied in an aqueous medium. It is therefore of interest to study the water in the immediate vicinity of the molecule to estimate how much is required for it to function and the minimum amount necessary for the protein to retain its three-diminsional structure. In a recent study (Lehmann & Zaccai, 1982) small angle neutron scattering was used to estimate the region around the enzyme papain that is inaccessible to ethanol and dimethyl sulfoxide. It was found that a large part of the protein surface was directly in contact with the probe molecules. The most simple and direct explanation of this is that both ethanol and dimethyl sulfoxide are small molecules that have the ability for either hydrogen-bond donation or hydrogen-bond acceptance similar to water and can thus replace this in the immediate environment of the macromolecule. Following this observation it was decided to do further studies of the same kind on ribonuclease A, and in addition to include glycerol as probe molecule, as this has been used recently in a study of preferential hydration of proteins in glycerol–water mixtures (Gekko & Timasheff, 1981). This not only allowed the comparison of observations using two different techniques, which in principle should lead to similar results (Eisenberg, 1981), but also provided the opportunity to compare the behavior of two solvent molecules of similar nature but of different size.

Materials and Methods

Ribonuclease A preparation X A was from Sigma (lot 39C-8035); it was dialyzed 3 times in a D_2O buffer (50 mM phosphate and 100 mM NaCl, pD 7.2) for a total period of 2 days at room temperature. Transmission measurements on the last buffer using neutrons showed the D_2O content to be higher than 99%. The probe molecules CH_3CH_2OD and $CH_2ODCHODCH_2OD$ were purchased from the Service des Molécules Marquées, CEA, Gif-sur-Yvette, and were enriched more than 99% and 97% in D, respectively. The glycerol contained less than 1% water. Mixing to the required volume percentages was done in microliter quantities using high-precision pipettes. For glycerol a mixture of glycerol and buffer was prepared before mixing with the sample in order to reduce the viscosity. The mixing ratio for sample and buffer was checked by using neutron transmission measurements, which are very sensitive to the hydrogen content. For the

† From the Institut Laue-Langevin, 156X, 38042 Grenoble Cedex, France. *Received August 4, 1983.*

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