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Real-time solvent exchange studies of the imino and amino protons of yeast phenylalanine transfer RNA by Fourier transform NMR

(conformational stability/tertiary hydrogen bonding/proton–deuteron replacement)

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ABSTRACT **Real-time solvent exchange measurements using Fourier transform NMR at 270 MHz are presented. By means of the fast gel filtration column techniques originally developed for tritium exchange experiments, we were able to replace the solvent of a tRNA sample from an ^1H_2O to an ^2H_2O buffer and obtain a useful spectrum in 2–5 min. At 15°C, there are 5 ± 1 lowfield (−11 to −15 ppm relative to 2,2-dimethyl-2-silapentane-5-sulfonate) imino protons with exchange half times of minutes to hours. In addition, the m^7G-46 C(8) proton and several amino protons are observed to exchange with similar rates. Analogous studies on unfractionated yeast tRNA suggest that such a class of slowly exchanging imino protons is present in several tRNAs, and that the activation energy for exchange is small [≈5 kcal/mol (21 kJ/mol)]. We speculate that these imino resonances arise from D-stem protons and that their slow exchange reflects stabilization by the numerous tertiary interactions involving this stem and the Mg^{2+} bound at the P-10 bend.**

The measurement of hydrogen exchange rates has become an important tool for studying macromolecular conformation and dynamics. The rate with which a labile proton exchanges with solvent can be greatly affected by the rate of structural fluctuations that expose such a proton to solvent and therefore can reflect dynamic aspects of conformation (1, 2). The most widely used approach to this problem has been the use of $^3H/^1H$ exchange gel filtration methods (1, 2); model studies with double helical polynucleotides indicate that at low temperatures both imino and amino protons exchange with rates slow enough to be detected by this technique (3–7). With tRNA, more slowly exchanging protons were observed than could be accounted for by the number of imino and amino hydrogens predicted from the secondary structure, and these extra protons were assigned to tertiary structure (4, 8–10). More recently, stopped-flow ultraviolet spectroscopy has been introduced into hydrogen exchange studies of nucleic acids (11, 12). This technique is applicable to much wider rate and temperature ranges than the tritium exchange methods. However, neither of these methods can make an unambiguous assignment of the observed rates to specific hydrogens in the structure.

There is a clear NMR spectral differentiation between imino and amino protons of nucleic acids (13), and studies on purified tRNAs have made progress in identifying the imino proton resonances with specific hydrogens in the secondary and tertiary structure (14, 15). Using pulsed Fourier transform NMR (FT NMR), we have studied the relaxation rates of the imino proton resonances of tRNA. At certain temperatures, within about 10°C of a melting transition, these rates are a direct measure of out-exchange (16, 17), and they are particularly useful in describing the melting transitions in tRNA (18). However, this method cannot be used to measure exchange rates that are less than about 10 sec^{-1} (17).

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The results presented in this paper extend our measurements of exchange to rates below about 0.005 sec^{-1}. The experiments are a combination of the fast gel filtration technology of the tritium exchange experiments and FT NMR. Similar experiments using continuous wave NMR revealed that measurement of out-exchange was not possible by this method (19). However, the increased sensitivity of pulsed FT NMR and the higher degree of solvent replacement have allowed us to demonstrate the ease and usefulness of these types of experiments.

MATERIALS AND METHODS

Yeast $tRNA^{Phe}$ was purchased from Boehringer Mannheim (lot 1038130) and used without further purification or was prepared as previously described (16). All $tRNA^{Phe}$ accepted at least 1.6 nmol of Phe per A_{258} unit of tRNA. The unfractionated yeast tRNA was purchased from Boehringer Mannheim.

The lyophilized tRNA was dissolved in an H_2O buffer containing 15 mM $MgCl_2$, 0.1 M NaCl, and 10 mM Na cacodylate (pH 7.0). A Sephadex G-25 column (5.0 × 0.35 cm; negatively charged sulfoethyl-Sephadex) was equilibrated with the same buffer in a 99.8% ^2H_2O solvent. The column was jacketed and maintained at 0°C with ice water. A typical exchange run was started by layering 50–100 μl of a 100% H_2O solution containing tRNA at 75–150 mg/ml, 15 mM $MgCl_2$, 0.1 M NaCl, and 10 mM Na cacodylate (pH 6.8) on top of the resin. A brief application of pressure by N_2 flow [2–3 pounds/$inch^2$ (14–21 kPa)], to run the tRNA solution into the resin, was quickly followed by ice-cold 99.8% ^2H_2O buffer, and pressure was again applied to elute the tRNA. A precise determination of the time required for tRNA to pass through the column was made by running several crude tRNA samples and monitoring the absorbance at 258 nm. Fractions used in the exchange measurements could be collected between 30 sec and 1.5 min after loading the column. For the NMR experiments, predetermined fractions (total volume 180–200 μl) were collected and quickly transferred to an ice-cold microcavity NMR tube (Wilmad no. 508-CP). The final tRNA concentration was 400–500 A_{258} units/ml. The spectrometer was preadjusted on a sample of similar characteristics such that locking the spectrometer was the only requirement before beginning data collection. Zero time for the NMR experiments was set as the time that the tRNA entered the Sephadex G-25 column. In preliminary experiments, the first analyzable spectrum (usable signal-to-noise ratio) could be obtained within 2–3 min after the tRNA entered the Sephadex G-25 column. Replacement of ^1H_2O by ^2H_2O upon passage of the tRNA solution through the column was

Abbreviations: FT NMR, Fourier transform NMR; FID, free induction decay.

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verified by integrating the ^1H_2O resonance of such a sample and comparing it to the ^1H_2O integral of a sample containing 95% ^1H_2O. Less than 3% residual ^1H_2O was detected in the exchanged sample.

The spectra were obtained as described (16); a special observation pulse that flips the imino or aromatic and amino protons without flipping solvent protons was used. All spectra were normalized before plotting to compensate for the different number of free induction decays (FIDs), except where noted.

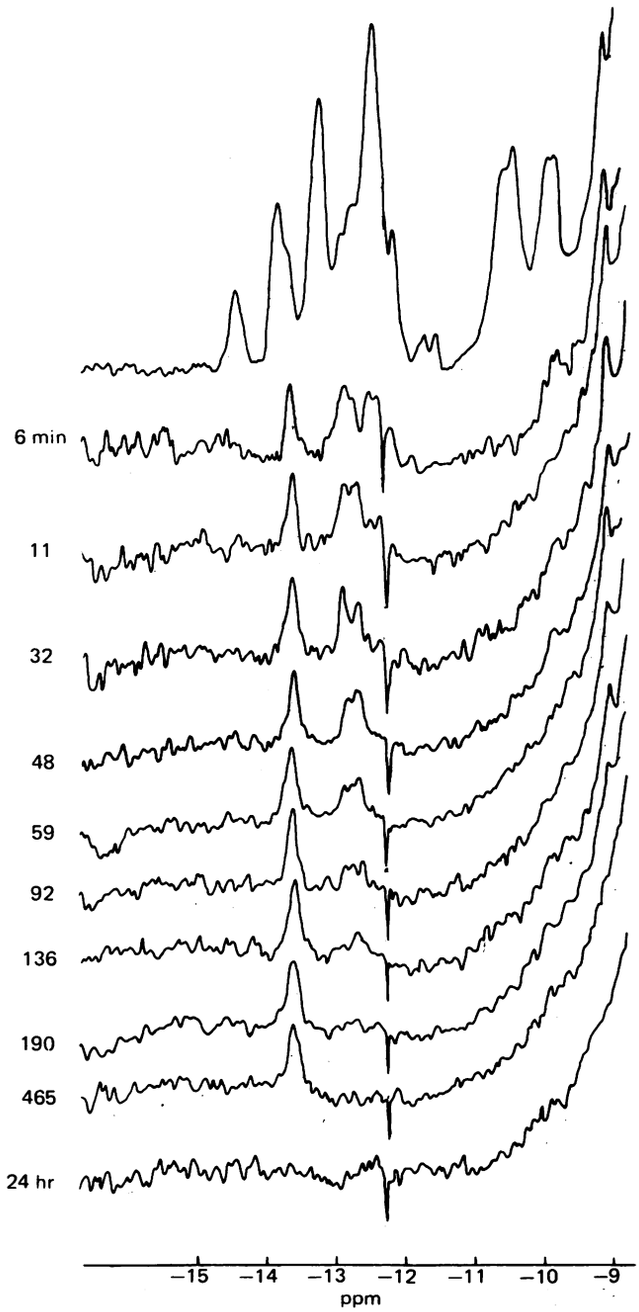

FIG. 1. Real-time exchange of protons for deuterons as monitored by the imino proton resonance region of yeast $tRNA^{Phe}$. All spectra were taken with the $tRNA^{Phe}$ in 15 mM $MgCl_2$/0.1 M NaCl/10 mM Na cacodylate (pH 7.0) at 15°C. The top spectrum was taken in a 95% ^1H_2O/5% ^2H_2O solvent; the rest were taken in a >97% ^2H_2O solvent. The spectra were taken at the times indicated after rapid solvent replacement. Each spectrum up to 35 min was the result of 1600 FIDs, whereas the spectra taken after 35 min were the result of 3200 FIDs. The tRNA concentration was 0.8 mM. The bottom spectrum was taken after the sample had been left at room temperature for 24 hr. In this and the following figures, ppm are relative to 2,2-dimethyl-2-silapentane-5-sulfonate.

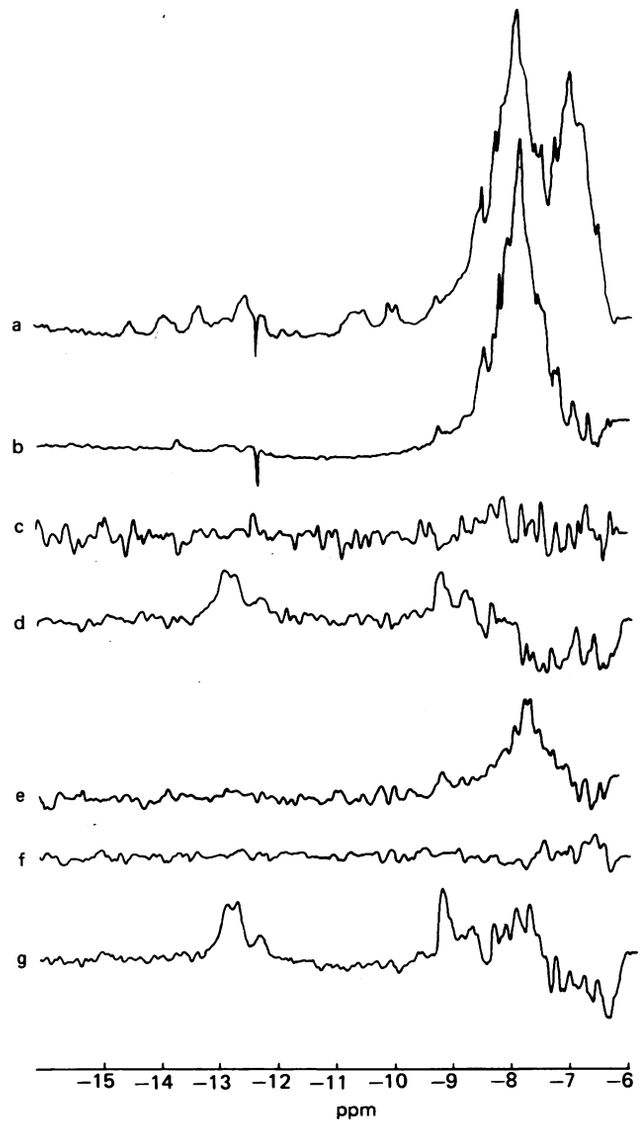

FIG. 2. Computer difference spectra showing the time dependence of exchange for the imino, amino, and aromatic protons in yeast $tRNA^{Phe}$ at 15°C. Spectrum a, 95% ^1H_2O/5% ^2H_2O, normal spectrum. Spectrum b, 6 min after solvent replacement, normal spectrum. Spectrum c, 6-min spectrum minus 11-min spectrum. Spectrum d, the sum of 6-, 11-, 16-, and 21-min spectra minus the sum of 190- and 201-min spectra. Spectrum e, the sum of 190- and 201-min spectra minus the sum of 323- and 334-min spectra. Spectrum f, the sum of 323- and 334-min spectra minus the sum of 456- and 465-min spectra. Spectrum g, the sum of 6-, 11-, 16-, and 21-min spectra minus the sum of 456- and 465-min spectra. Spectra c–g are plotted to be 4× the intensity of spectra a and b. The buffer conditions and spectral conditions are the same as for Fig. 1.

RESULTS

Fig. 1 presents a real-time out-exchange profile of the imino protons of yeast $tRNA^{Phe}$ at 15°C as monitored by FT NMR. The first spectrum recorded at 6.5 min after solvent replacement shows several imino protons, which resonate at −13.64, −12.9, −12.65, and −12.5 ppm from 2,2-dimethyl-2-silapentane-5-sulfonate, with exchange times long enough to be detected by these means. In addition, there is a resonance at −9.14 ppm that is likely to be the C(8) proton of m^7G-46 (20) which also exchanges over the same time range. The bottom spectrum,

Table 1. Hydrogen exchange times for yeast $tRNA^{Phe}$ at 15°C

Resonance, ppm	Integral, no. of protons	$t_{1/2}$, min	Type of proton
−13.64	1.5	>465	A·U imino
−12.9 to −12.65	≈3	76	G·C imino
−12.2 to 12.5	≈1	Gone by 10	G·C imino
−9.14	—	100–200	Purine C(8)

taken after leaving the sample at room temperature for 24 hr, shows that the resonance at −13.64 ppm is indeed due to an exchangeable proton.

The data summarized in Table 1 indicate that there are 5 ± 1 ring nitrogen protons in yeast $tRNA^{Phe}$ with exchange times greater than 6.5 min at 15°C in the presence of Mg^{2+}. It is evident that there exists a large difference in the absolute rates, suggesting different environments for these hydrogens. Furthermore, the disappearance of a resonance at −9.14 ppm indicates that the C(8) proton of m^7G-46 has a much higher exchange rate than do other typical purine C(8) protons of $tRNA^{Phe}$ (21).

Proton exchange in the amino-aromatic region (−6.0 to −9.0 ppm) can best be analyzed by the use of difference spectra because of the great number of resonances in this region. In Fig. 2, the time dependences of exchange for all the exchangeable protons in yeast $tRNA^{Phe}$ are presented as difference spectra. Spectrum c indicates possible exchange at −12.5 ppm and throughout the amino region at the earliest detection times; however, the noise in this spectrum makes it difficult to analyze. The signal-to-noise ratio can be increased at the later times by summing several spectra before taking the difference. This has been done for spectra d–g. Over the period of time that allows complete exchange of the G·C protons resonating between −12.5 and −12.9 ppm there are also a limited number of amino protons undergoing exchange (spectrum d). Spectrum e indicates that exchange is still taking place in the amino-aromatic region even though all but approximately one proton resonance has disappeared in the imino region. Spectrum g presents the total exchange observed over 465 min, and a small but significant amount of exchange has taken place involving the C(8) proton of m^7G-46 and several imino and amino protons.

We have extended these studies to unfractionated yeast tRNA over a 10°C temperature range. Fig. 3 is representative of the results and was taken at 19°C. The first time point was obtained at 2.0 min after the column had been loaded, and complete exchange, under these conditions, took place by 34 min. At 10°C, we did not observe a significant increase in the number of nonexchanged imino protons in the first time point compared to the behavior at 19°C; however, complete exchange required 60 min. At 15°C, 45 min was required for complete exchange. Thus, it is obvious that the slow exchange of imino protons in yeast tRNA is a general feature, and that temperature-dependent studies are quite feasible.

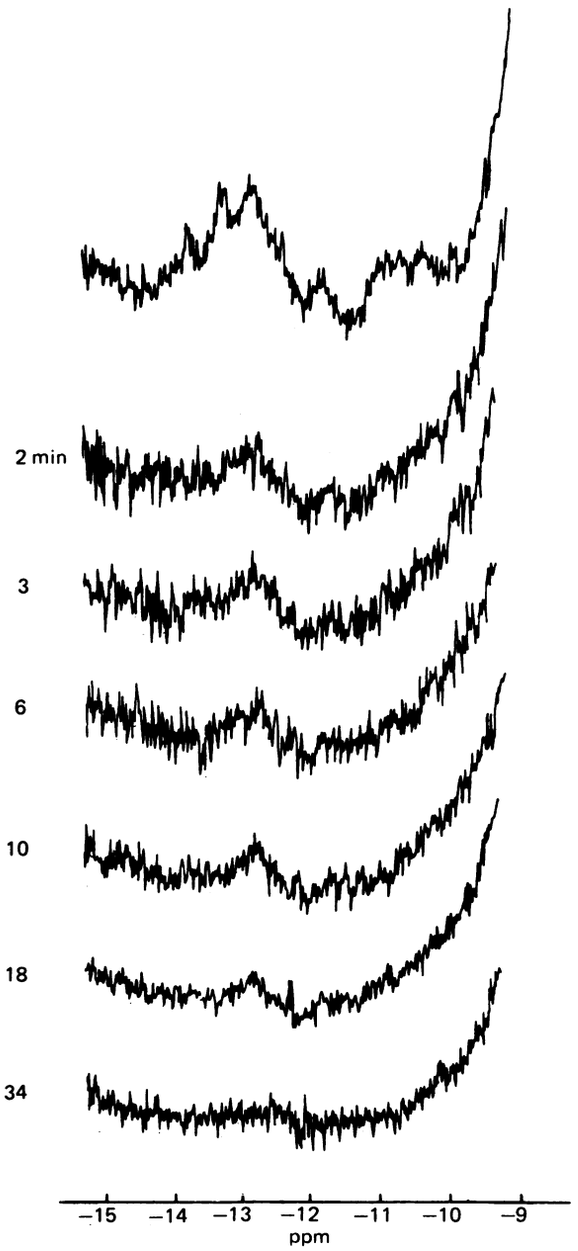

FIG. 3. Real-time out-exchange of the imino protons of crude yeast tRNA as monitored by observing the lowfield resonance region. The top spectrum was taken in 95% ^1H_2O/5% ^2H_2O. The other spectra were taken at the times indicated after rapid solvent replacement to a >97% ^2H_2O solvent. The buffer conditions are the same as for Fig. 1 at 19°C. The top spectrum and the 34- and 18-min spectra are the result of 650 FIDs. The spectra at 2, 3.3, and 5.8 min are the result of 160 FIDs, and the 10.2-min spectrum is from 320 FIDs.

DISCUSSION

This paper has demonstrated the existence of protons (both imino and amino) in yeast $tRNA^{Phe}$ and unfractionated tRNA that are in slow enough exchange with solvent (<0.005 sec^{-1}) to be detected by a combination of fast gel filtration methods (4) and FT NMR. The advantage of these experiments is that the measured rates can often be correlated with specific hydrogens in the structure of tRNA, and moreover, easy distinction can be made between imino and amino protons.

On the basis of both ring current predictive schemes and relaxation rate melting data, the proton resonance at −13.64 ppm can be assigned to one of the three secondary A·U base pairs, 5, 12, or 52 (15, 18). Each of these base pairs is derived from the secondary structure, and it is not immediately apparent why only one would have such extremely slow exchange properties ($t_{1/2}$ > 465 min). Of these three base pairs, A-23·U-12 is unusual in that it is also involved in tertiary H bonding interactions (22–24). In addition, the phosphate of U-12 is part of the binding pocket for Mg^{2+} at the P-10 bend (25–27) and Mg^{2+} would likely be occupying the binding site under the conditions of our experiments. This area of tRNA structure forms a central core of the molecule, and the tertiary interactions involve a number of base–base, base–backbone, and backbone–backbone interactions. There are seven secondary

A·U base pairs in yeast $tRNA^{Phe}$; those of the D, TΨC, and anticodon stems are in more or less similar environments with respect to the secondary base pairing (i.e., internal and surrounded by G·C base pairs). Furthermore, it has been shown that the A·U base pairs of the acceptor stem correspond to one of the least stable sections of the structure (18, 28). Thus the most reasonable explanation for the extremely slow exchange of the proton responsible for the resonance at −13.64 ppm is that it is the imino proton of A·U-12. The slow exchange can be explained by the involvement of A-23 with A-9 in a poly(rA)-type pairing (22–24) and of the interaction between U-12 O(1′) and C-11 O(2′) (24). In addition, a Mg^{2+} is hydrogen bonded through its coordination sphere to the four phosphates, 8, 9, 11, and 12 (25–27).

Three of the four slowly exchanging G·C imino protons responsible for the resonances between −12.5 and −12.9 ppm can be assigned to the other secondary base pairs of the D stem (G·C-10, G·C-11, and G·C-13) by the same arguments. That is, there is at least one type of tertiary H bond (base–base, base–backbone, or backbone–backbone) to each G·C base pair of the D stem (22–24) and the Mg^{2+}-binding site discussed above also involves the phosphate of C-11 (25–27). This conclusion is also consistent with melting data and ring current predictions (15, 18). It is not clear, however, why A·U-12 has such a long exchange time compared to the other D-stem protons.

One other G·C base pair proton shows anomalously slow exchange, namely the proton that accounts for the resonance at −12.9 ppm. This resonance has been assigned to the imino proton of the tertiary interaction G-19–C-56 on the basis of ring current calculations and relaxation rate melting data (16). The base pair G-19·C-56 was also found to be one of the most stable tertiary interactions in yeast $tRNA^{Phe}$ (18, 28). Like the slowly exchanging protons from the D stem, G-19–C-56 is involved in other stabilizing interactions. The phosphate and ribose of G-19 are involved in tertiary H bonding, and the bases stacked upon G-19–C-56 (i.e., G-57 and G-18–Ψ-55) are also heavily involved in tertiary H bonding interactions (22–24). In addition, there are two Mg^{2+}-binding sites involving G-19–C-56 and the nearby bases (25–27). Thus, there is ample evidence to support the suggestion that Mg^{2+} binding and tertiary structure are sufficient to impede the exchange of some H bonded protons of bases involved in secondary and tertiary interactions.

This conclusion is further substantiated by the results with yeast unfractionated tRNA. We find that there is a class of protons whose exchange is much slower than the majority of H bonded imino protons (Fig. 3). The number of protons in this class does not greatly increase from 20°C to 10°C even though the rates decrease by about a factor of 2. This suggests that the results observed with $tRNA^{Phe}$ are true for many yeast tRNAs, and most probably for those with a tertiary structure similar to that of yeast $tRNA^{Phe}$. In other words, the D-stem imino protons and G-19–C-56 (for yeast $tRNA^{Phe}$) form an anomalously slow exchange class due to the great number of tertiary interactions to these base pairs and the presence of tightly bound Mg^{2+}.

A factor of 2 change in rate was observed for the imino protons of crude tRNA over a 10°C temperature range. This is in contrast to purified yeast $tRNA^{Phe}$ at temperatures near a melting transition, where a 10-fold change in rate was observed over a 10°C temperature range (17). Thus a different mechanism of exchange appears to be important at low temperature (well below a melting transition) than is observed near melting temperatures.

For yeast $tRNA^{Phe}$, there are also a number of slowly exchanging amino protons with rates similar to those of the G·C imino protons (Fig. 2). The similarity of the rates could be fortuitous, but the number of protons exchanging in about 3 hr and resonating in the −6 to −9 ppm region is comparable to the number of amino protons expected from three G·C base pairs (Fig. 2, spectrum d). There are about six such amino proton resonances in this region (Fig. 2, spectrum d), as would be expected if the three cytidine N(4) amino groups (2, 7) exchange slowly, and if the corresponding guanosine N(2) amino protons exchange at a faster rate. Such a result is not unexpected because it is known that the G amino groups can exchange without disruption of the base pair and that both protons show identical rates in polynucleotides (2, 7). Because it is necessary that the base pair be disrupted for A and C amino groups to exchange (2, 6, 7), it is possible that a common structural event could control the observed exchange rates of imino and amino protons of cytidine and adenosine in a Watson–Crick base pair. However, this interpretation is complicated by the observed exchange of other protons resonating in the −6 to −9 ppm region (Fig. 2, spectrum e) in the absence of imino proton exchange, and is, therefore, speculation.

It is known that the purine C(8) proton can exchange with solvent in several hours at 80°–100°C (29). When the purine is involved in base pairing interactions in $tRNA^{Phe}$, the rate can be reduced to as little as 1/5th–1/30th (21). Thus, the slowly exchanging protons whose resonances are observed in the −6.0 to −9.0 ppm region (Fig. 2) can all be assigned to amino protons.

However, there is one exception to this conclusion. The slowly exchanging proton that resonates at −9.14 ppm can be assigned to the C(8) proton of m^7G-46 (20). The presence of the positive charge in m^7G due to methylation at N(7) makes the C(8) proton extremely labile, with a $t_{1/2}$ of 5.5 min at pH 4.5, 28°C, and much faster at pH 7.0 (30). In $tRNA^{Phe}$, m^7G-46 is involved in a tertiary base pair with G-22 and is stacked between A-21 and A-9 (22–24). Therefore the exchange rate observed ($t_{1/2} \approx$ 100–200 min) is completely consistent with the assignment of the resonance at −9.14 ppm to the C(8) proton of m^7G-46.

In conclusion, these observations show that NMR measurements of proton solvent exchange rates, on the order of a few minutes, are feasible for macromolecules. These results provide a useful supplement to tritium exchange measurements at lower concentrations and to NMR relaxation measurements, which reflect exchange only at higher temperatures. In the case of tRNA, our real-time exchange results show that there is considerable heterogeneity in the low-temperature exchange rates of chemically similar protons and that the D stem is probably the most stable region of the structure at low temperatures and at high $MgCl_2$ concentrations. The rates are slow enough that it may be possible, by means of the influence on tRNA proton exchange, to study the effects on tRNA conformation of the various proteins known to interact with tRNA.

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