Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 4330–4333, July 1983
Biophysics

NMR study of slowly exchanging imino protons in yeast $tRNA^{Asp}$

(tertiary structure/nuclear Overhauser effect/deuteron labeling/buffer catalysis)

NARA FIGUEROA[a,b,c], GERARD KEITH[d], JEAN-LOUIS LEROY[a], PIERRE PLATEAU[a], SIDDHARTHA ROY[e,f], AND MAURICE GUERON[a,g]

[a]Groupe de Biophysique, Ecole Polytechnique, 91128 Palaiseau, France; [d]Institut de Biologie Moleculaire et Cellulaire, 15 rue Descartes, 67084 Strasbourg, France; and [b]Biophysics Graduate Program and [c]Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Communicated by Alfred G. Redfield, April 18, 1983

ABSTRACT **We have monitored the exchange of imino and amino protons by NMR after quick transfer of yeast $tRNA^{Asp}$ in ^2H_2O solvent. When the concentration of exchange-catalyzing buffer is not too high, one imino proton exchanges considerably more slowly than any other (e.g., 100 hr versus 4 hr for the second-slowest imino proton at 18°C in 15 mM Mg). This provides excellent conditions for identification, by the nuclear Overhauser effect, of the slowest exchanging proton, which we show to be the imino proton of the U-8·A-14 reverse Hoogsteen tertiary-structure base pair; other slowly exchanging protons are identified as imino protons from A·U-11 and G·Ψ-13. In preliminary experiments, we find that the exchange of these protons is catalyzed by cacodylate or Tris buffer. The lifetimes of two other imino protons, *ca.* 10 min at 28°C, are buffer independent. Slowly exchanging amino protons have also been observed. Correlation with the exchange of the uracil-8 imino proton suggests that they may be from adenine-14.**

tRNA molecules have a number of protons in slow exchange with solvent, whose lifetimes range between seconds and hours (1–4). Among these, one finds imino protons whose exchange, normally very fast in the free bases, has been slowed down because of hydrogen bonding or shielding from solvent (2). Conditions that affect the dynamic behavior of the molecule will influence the number as well as the rates of exchange of slowly exchanging protons. Several studies of proton exchange behavior of tRNAs have been carried out and a considerable amount of information has been obtained, mostly by the ^1H–^3H solvent replacement technique developed by Englander *et al.* (1, 2, 5). Recently, a method has been described that combines the resolution of tRNA proton NMR with ^1H–^2H solvent replacement (6). It provides the possibility of identifying the protons whose rates are being studied, therefore rendering the hydrogen exchange studies much more meaningful.

In the proton NMR spectra of tRNAs in H_2O, imino protons resonate between −8 and −16 ppm (7). In particular, this region contains a single resonance from each A·U or G·C Watson–Crick base pair and two (or possibly three) from each G·U or G·Ψ pair (Ψ, pseudouracil). If solvent is quickly changed from H_2O to ^2H_2O, the imino proton resonances will progressively disappear through exchange. This has been observed in crude tRNA as well as in yeast $tRNA^{Phe}$ (6), and similar experiments have been carried out with some proteins (8). The yeast $tRNA^{Phe}$ study reported the existence of about five very slowly exchanging imino protons, whose identity could not be confirmed, because good spectral assignments have only recently become available (9, 10). For yeast $tRNA^{Asp}$ also, a large part of the imino proton spectrum has now been assigned through a combination of transfer of saturation techniques with specific deuteration of the bases (11, 12). In this paper, we report the observation and identification of very slowly exchanging imino protons in yeast $tRNA^{Asp}$. Many are found in a single region of the molecule. A careful study by the nuclear Overhauser effect (NOE) and selective deuteration shows that the slowest exchanging imino proton is that of the tertiary base pair U-8·A-14. The exchange of this proton is buffer catalyzed.

MATERIALS AND METHODS

Preparation of Samples. Fully protonated yeast $tRNA^{Asp}$ was prepared as described (13) and its amino acid acceptance was 1,450 pmol/A_{260} unit. The C_8-deuterated tRNA was obtained from a side fraction of the benzoyl-DEAE-cellulose step described by Sanchez *et al.* (14). Integrity of tRNA was routinely checked by electrophoresis (12% polyacrylamide, 40 cm length). Samples of 100 A_{260} units of tRNA were treated with Chelex 100 in 0.1 M NaCl/10 mM Na cacodylate, pH 7.0, to remove all traces of divalent metal ions (15). The samples were then extensively dialyzed against 1 mM Na cacodylate (pH 7.8) and then against 0.5 mM Na cacodylate (pH 7.8); pH was checked and adjusted with NaOH if necessary. Chelex beads were always present in the dialysate. After dialysis, samples were lyophilized in conic glass tubes; resuspension in ever-decreasing volumes of H_2O and lyophilization resulted in a very small lump at the bottom of the tube.

Exchange Experiment. One hundred A_{260} units of material lyophilized as described above was suspended in 8 μl of H_2O buffer containing 0.1 M NaCl, 15 mM $MgCl_2$, 10 mM Na cacodylate, pH 7.0. At this stage, the mixture was paste-like. At 4°C, 150 μl of ^2H_2O buffer of identical composition was added and the solution was stirred vigorously with a pipette. This time was defined as $t = 0$. The sample was then placed in a NMR microcell (Wilmad 508 CP) and kept on ice until introduced in the spectrometer, which was pretuned with a similar sample. A spectrum could be obtained within 5 min of $t = 0$. The pD of the sample was 6.2, as determined from the chemical shift of the cacodylate methyl. (This is the midpoint of a separate titration of this species in ^2H_2O, the pD being the uncorrected value measured with a pH meter.) The final cacodylate concentration was calculated to be 14 mM.

NMR Methods. Spectra were measured at 276 MHz in a home-built Fourier transform spectrometer (16). The origin of the chemical shifts was 2,2-dimethylsilapentane-5-sulfonate, whose frequency is computed from that of the lock signal provided by ^2H_2O. The water signal was minimized by the use of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Ψ, pseudouracil; NOE, nuclear Overhauser effect.

[c]Present address: Institut de Biologie physico-chimique, 13 R. Pierre et Marie Curie, 75005 Paris, France.
[f]Present address: Bldg. 10-9 N313, NIADDK, NIH, Bethesda, MD 20205.
[g]To whom reprint requests should be addressed.

a (θ°_{-y};10τ;θ°_y;90$^\circ_y$;τ;90$^\circ_{-y}$) sequence (17) with $\theta^\circ = 5.7^\circ$ and τ = 125 μs at the resonant frequency of water. This sequence of strong pulses provides second-order suppression of H_2O excitation without base-line distortion. The repetition time was 0.25 s. In the NOE experiment, a selective inversion of the line at −13.9 ppm was carried out before the observation pulse. This was achieved by preirradiation, still at the H_2O resonant frequency, using a $(\theta^\circ_y;\tau;\theta^\circ_{-y})_n$ DANTE sequence (18), with θ° = 3°, τ = 200 μs, and n = 30. The phase alternation enables us to selectively invert a line that is offset from the irradiation frequency. The time between the end of the preirradiation sequence and the beginning of the observation pulse was 35 ms.

Thus, selective suppression of water and selective inversion of one spectral line were obtained using only *strong* pulses at a *single* radio-frequency.

RESULTS

Slowly Exchanging Protons. The results of an exchange experiment carried out with yeast $tRNA^{Asp}$ at 28°C are shown in Fig. 1. Several imino protons still remain 10 min after transfer from H_2O to ^2H_2O. Of these, only peaks B and I_2 are still present 40 min later and peak B can still be detected after 19 hr.

The lifetimes of the slowly exchanging protons are given in Table 1, together with proposed assignments. Peaks A, I_2, and L correspond to positions of proton resonances that have been unambiguously assigned by Roy and Redfield (11) and Roy *et al.* (12) using NOE techniques (Fig. 1). Peak B is at the position (−13.9 ppm) similarly assigned (11) to U-8·A-14. (We have repeated and confirmed the measurements leading to these assignments.)

Assignment of the Slowest Exchanging Proton. Among the slowly exchanging imino protons, peak B, assigned to U-8·A-14, has a uniquely long lifetime. We found it surprising that the slowest exchanging proton should be in a tertiary base pair. Considering also the possibility of saturation spillover to several peaks located near −13.9 ppm, we decided that the assignment should be strengthened as much as possible. In the case of a slowly exchanging proton, we have the possibility of carrying out NOE experiments in ^2H_2O. Because only one imino peak remains, spillover is then excluded and any observed NOEs must originate from the slow exchanging proton.

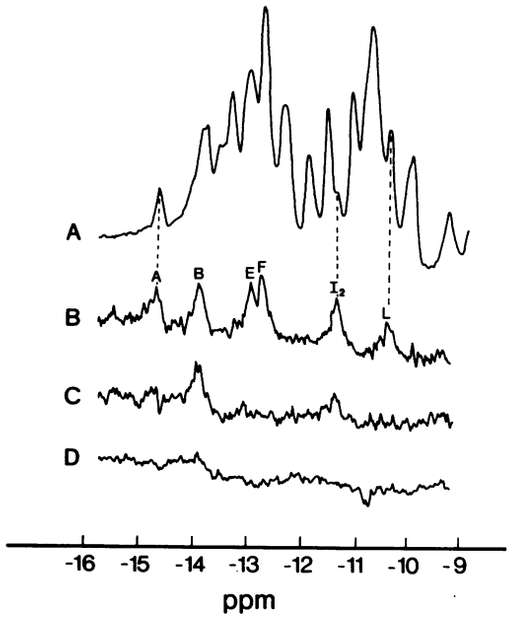

FIG. 1. Exchange experiment at 28°C. Traces: A, spectrum in H_2O (6,000 scans); B, 10 min after solvent change (900 scans); C, 38.5 min after solvent change (900 scans); D, 19 hr after solvent change (4,500 scans). Spectra were broadened by 8 Hz and normalized for the number of scans. Solution conditions were 0.1 M NaCl/14 mM Na cacodylate/15 mM $MgCl_2$, pD 6.2/tRNA, *ca.* 1 mM. Peak identifiers correspond to those used in ref. 12.

Table 1. Slowly exchanging imino protons in yeast $tRNA^{Asp}$

Peak	Position, ppm	Assignment	Lifetime (min) 28°C*	18°C*	18°C†
A	−14.7	A·U-11	30	35	NS
B	−13.9	U-8·A-14	630	6,080	200
E	−12.96	?	15	65	60
F	−12.75	?	10	65	60
I_2	−11.36	G·Ψ-13	35	260	NS
L	−10.38	G·Ψ-13	10	20	NS

NS, not seen (lifetime, <10 min).
*In 14 mM Na cacodylate, pD 6.2/0.1 M NaCl/15 mM $MgCl_2$.
†In 14 mM Na cacodylate, pD 6.2/0.1 M NaCl.

Original assignment of the −13.9-ppm peak to U-8·A-14 (11, 12). If one assumes for yeast $tRNA^{Asp}$ a structure similar to that of yeast $tRNA^{Phe}$, two reverse Hoogsteen base pairs are expected to form: U-8·A-14 and T-54·A-58. Reverse Hoogsteen base pairs give a NOE from the imino proton to adenine C(8), whereas the NOE in a Watson–Crick base pair is to C(2). Using tRNA deuterated on the C(2) of adenine (12) and tRNA deuterated on the C(8) of purines (11), two reverse Hoogsteen base pair imino protons were indeed found, and each was assigned. The assignment of the −13.9-ppm peak to U-8·A-14 was based on the following: (*i*) a sharp NOE to −7.8 ppm, which vanishes on C(8) deuteration (this proves the reverse Hoogsteen structure) and (*ii*) small (inter-base-pair) NOEs from −13.9 to −10.3 and −11.54 ppm, characteristic of a G·U or G·Ψ base pair. Of the four such base pairs in the molecule, only one is close to a reverse Hoogsteen base pair, namely G·Ψ-13, close to U-8·A-14.

NOE experiments in ^2H_2O. We first carried out an experiment with tRNA fully protonated on C(8). The result of irradiation at −13.9 ppm is shown in Fig. 2 (trace A). Prior to the experiment, the sample was incubated for 24 hr at 15°C, after solvent replacement, to allow exchange of all imino protons except that at −13.9 ppm. There is a sharp NOE at −7.8 ppm and a broad one at −8.8 ppm, in agreement with the earlier measurements in H_2O. (Another broad peak is observed at −7.9 ppm. This is discussed below.)

All NOEs decreased in intensity together with the −13.9 ppm peak (lifetime, ≈3 days at 20°C). As is to be expected in ^2H_2O, there were no NOEs to the (exchanged) imino protons of G·Ψ-13 at −10.3 ppm. These results confirm that the NOEs at −7.8 ppm and −8.8 ppm arise from irradiation of one and the same proton at −13.9 ppm and thus support the earlier assignment to U-8·A-14. In consequence they show that U-8·A-14 must contain the slowest exchanging proton. Another observation that supports the U-8·A-14 assignment is the persistence of the slow exchange at 18°C in the absence of magnesium (lifetime, 200 min). Under these conditions, the nearby resonance of A·U-7 is melted (12), so that A·U-7 is excluded as a candidate for the slow exchanger.

Lastly, we have carried out the ^2H_2O version of the NOE experiment with C(8)-deuterated tRNA. The results are shown in Fig. 2 (trace B). The NOE to the narrow peak at −7.8 ppm has nearly vanished, whereas the broad peaks at −7.9 and −8.8 ppm appear unchanged. This agrees with the earlier experiment in H_2O and thus supports the earlier conclusion that the −13.9-ppm peak is from a reverse Hoogsteen base pair (a bet-

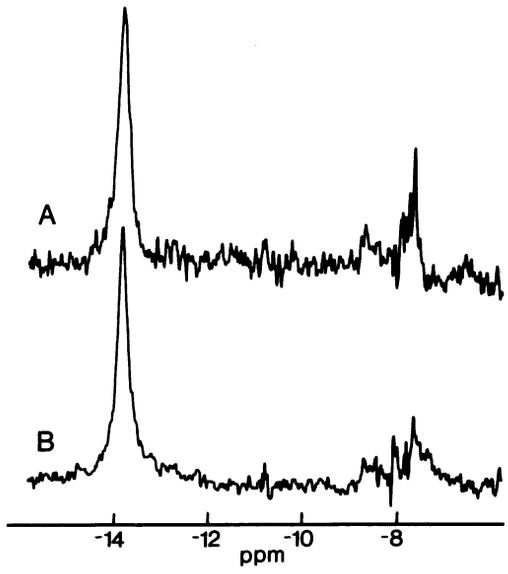

FIG. 2. NOE experiment at 20°C in ^2H_2O. Solution conditions were as in Fig. 1. The figures show differences between spectra (data not shown) obtained by preirradiating alternately at −16.13 ppm and −13.9 ppm. Traces: A, fully protonated tRNA; B, tRNA deuterated on C(8) of purines. The narrow peak at −7.8 ppm in trace A is absent in trace B.

ter signal/noise ratio would help).

Carried out on a spectrum containing a single imino proton, the experiments in ^2H_2O support and strengthen the earlier assignment of the −13.9-ppm peak to U-8·A-14, and they show that U-8·A-14 is the slow exchanger. They demonstrate the use of the NOE in ^2H_2O for the assignment of an imino proton resonance.

Slowly Exchanging Amino Protons. According to Teitelbaum and Englander (19), exchange of the amino protons of adenine or cytosine requires attack of one of the amino protons by a general base and simultaneous protonation at the imido position [N(1) of adenine, N(3) of cytosine]. Since these imido positions are hydrogen bonded in Watson–Crick base pairs and cannot then be protonated (19), we were induced to search for slowly exchanging amino protons. A difference spectrum from an exchange experiment carried out at 35°C is shown in Fig. 3. The lifetime of the (reverse-Hoogsteen) U-8·A-14 imino proton at −13.9 ppm is 10 min. Some protons in the amino region exchange even more slowly (300–1,000 min). Remarkably, two of these, at −7.9 and −8.8 ppm, correspond to the broad NOEs from the U-8·A-14 imino (Fig. 2), suggesting that they may be amino protons from adenine-14.

At first sight this is surprising: in the reverse-Hoogsteen U-8·A-14, the N(1) imido position of adenine is not blocked by hydrogen bonding to uracil-8, and one would therefore expect the adenine-14 amino protons to exchange quickly. But the recent refinement of crystallographic data (D. Moras, personal communication) shows that, in contradistinction to yeast $tRNA^{Phe}$, adenine-21 of yeast $tRNA^{Asp}$ is placed so that it might block access to the amino group of adenine-14. Both amino protons of adenine-14 would then be inaccessible to a general base, and their exchange would thus be inhibited.

Lastly, it should be pointed out that amino protons could be broader in a proton than in a deuteron environment. This could explain why the −7.9 line was not seen in the original H_2O experiment.

Buffer Effects. The effect of buffer on the exchange rate is a clue to the mechanism of exchange (19). In preliminary studies, we found that the exchange rates of the unidentified imino

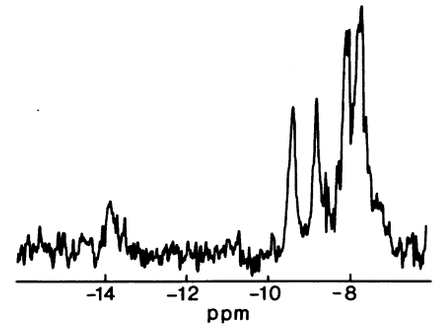

FIG. 3. Amino proton exchange experiment at 35°C. The spectrum shown is the difference between spectra accumulated at early (180–400 min) and late (900–1,120 min) times after solvent exchange. The initial waiting time results in disappearance of most amino protons and all imino protons except that of A·U-8 at −13.9 ppm, whose intensity corresponds to 0.2 proton (the lifetime, measured independently, is 150 min). The amino proton lifetimes are in the range of 300 min (at −9.5 ppm) to 1,000 min (at −7.9 ppm) under the same conditions. Two peaks occur at the same positions (−7.9 and −8.8 ppm) as the broad NOEs of Fig. 2 (trace B). They may correspond to the amino protons of adenine-14. Conditions were 0.1 M NaCl/10 mM Na cacodylate/15 mM $MgCl_2$/tRNA, *ca.* 1 mM; recurrence time, 1.2 s.

protons at −12.96 and −12.75 ppm (Fig. 1) are not sensitive to the concentration of Tris or cacodylate buffer. In contrast, the exchange rates of the imino protons of U·G-10, A·U-11, G·Ψ-13 and U-8·A-14 are increased by these buffers. Furthermore, slow exchange of the imino proton of U·G-10 at −11.8 ppm is observed when the cacodylate buffer concentration is reduced to 0.1 mM. The lifetime of the imino proton of U-8·A-14, extrapolated to infinite buffer concentration, is only 5 min at 20°C.

DISCUSSION

Existence of a Region of Slowly Exchanging Protons. If one considers the tertiary structure of yeast $tRNA^{Asp}$ (using yeast $tRNA^{Phe}$ as a model; Fig. 4), it appears that the assigned slowly

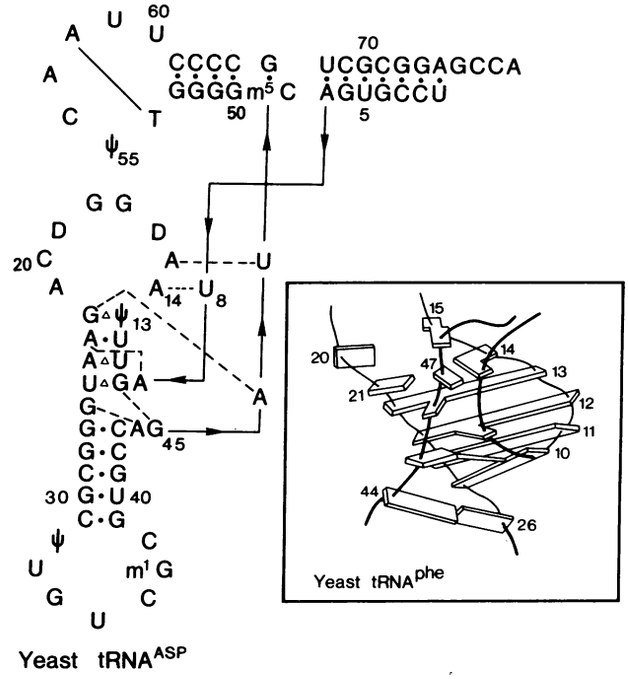

FIG. 4. Nucleotide sequence of yeast $tRNA^{Asp}$ [presented as in Kim *et al.* (20)] and tertiary representation of the D-stem region in yeast $tRNA^{Phe}$.

exchanging protons are found in a single domain of stacked base pairs and triplets. The U-8·A-14 base pair, which forms the so-called "augmentation" of the D stem, stacks on the first base pair of the D stem, G·Ψ-13, which is also a slowly exchanging one, and is itself stacked on the next base pair of the D stem A·U-12. A·U-12, which is not slowly exchanging, stacks on A·U-11 (which is), itself stacked on U·G-10 (marginally slow; see below), the last base pair of the D stem.

These observations show that exchangeable protons in the augmented D-stem domain are remarkably protected against proton exchange. However, to go beyond this qualitative statement, one must investigate the *mechanism* of this protection. If proton exchange in the open state is faster than return to the base-paired state, the measured exchange rate is equal to the base-pair opening rate (the inverse of the base-pair lifetime). If the converse is true—i.e., if return to the base-paired state occurs faster than proton exchange in the open state—the observed exchange rate is smaller than the opening rate (19). Since the exchange rates of the unidentified imino protons at −12.96 and −12.75 ppm (Fig. 1) are not sensitive to buffer, they likely reflect base-pair opening. In contrast, the exchange rates of the imino protons of U·G-10, A·U-11, G·Ψ-13, and U-8·A-14 are limited by chemical exchange.

The Slowest Exchanging Proton. Whereas an isolated uridine exchanges its imino proton with solvent in a small fraction of a second, the imino proton of U-8·A-14 exchanges in 10 hr at 28°C and 100 hr at 18°C in 14 mM cacodylate buffer/15 mM Mg. Such times are 20 times as large as those for the second-slowest imino proton of yeast $tRNA^{Asp}$.

The dependence on buffer concentration shows that the observed exchange rate is much smaller than the rate of base-pair opening: 0.2/min at 20°C. Hence, when the base pair is open, it usually closes back before the proton has time to exchange.

We cannot currently explain this phenomenon, but we note for comparison that, in yeast $tRNA^{Phe}$, U-8·A-14 exchanges rapidly (6). Two features may contribute to this difference: one is the absence of base 47 in yeast $tRNA^{Asp}$. This may improve the stacking of U-8·A-14 on A-15·U-48. The other is the position of adenine-21, which was invoked above to explain the slowly exchanging amino protons at −7.9 and −8.8 ppm, to which the uracil-8 imino proton is NOE connected.

The buffer effect on the lifetime of the uracil-8 imino proton offers a means to explore the properties of the U-8·A-14 base pair.

Comparison with Other tRNAs. Comparative studies of the slowly exchanging protons will be reported elsewhere. We mentioned above that in yeast $tRNA^{Phe}$ U-8·A-14 is not a slowly exchanging proton. In this tRNA species, a very slowly exchanging proton falls at a position assigned to G·C-11 (6, 9, 10); two slow but shorter lived spectral peaks are assigned to G·C-13 and m^2G·C-10 (10). Thus, in this tRNA also, exchange of some D-stem imino protons is slow. In this structural region, the two tRNAs differ with respect to the position of adenine-21, as noted, and also regarding G-26·A-44. In yeast $tRNA^{Phe}$, this base pair is m^2_2G-26·A-44, and it is twisted because of the two methyl groups (20).

Recently, Heerschap *et al.* (9) have observed the NOEs associated with U-8·A-14 and with some protons of the D stem of yeast $tRNA^{Phe}$, in 5 mM Mg^{2+} at 45°C, whereas NOEs of protons from other parts of the molecule have vanished under these conditions. We would expect a connection between persistence of the NOE and slow proton exchange, whether the latter is due to a long base-pair lifetime or to fast closure of the open structure.

Imino protons as long-lived as the ones described in this study have not been reported in the classic hydrogen–tritium exchange studies of nucleic acids. A possible explanation is that they could have been overlooked due to the experimental protocol (too short a preincubation in ^3H for example). Such protons could exist not only in tRNAs but also in other RNAs with a highly complex tertiary structure such as ribosomal RNAs.

We wish to thank Prof. A. G. Redfield for guidance and Dr. D. Moras for communication of unpublished results. S.R. acknowledges partial support from U.S. Public Health Service Grant GM 20168. N.F. acknowledges support from the Ecole Polytechnique.

1. Englander, S. W. & Englander, J. J. (1965) *Proc. Natl. Acad. Sci. USA* **53**, 370–378.
2. Englander, J. J., Kallenbach, N. R. & Englander, S. W. (1972) *J. Mol. Biol.* **63**, 153–169.
3. Goldstein, R. N., Stefanovic, S. & Kallenbach, N. R. (1972) *J. Mol. Biol.* **69**, 217–236.
4. Webb, P. K. & Fresco, J. R. (1973) *J. Mol. Biol.* **74**, 387–402.
5. Englander, S. W. & Englander, J. J. (1978) *Methods Enzymol.* **49G**, 24–39.
6. Johnston, P., Figueroa, N. & Redfield, A. G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3130–3134.
7. Schimmel, P. R. & Redfield, A. G. (1980) *Annu. Rev. Biochem.* **9**, 181–221.
8. Wüthrich, K. & Wagner, G. (1979) *J. Mol. Biol.* **130**, 1–30.
9. Heerschap, A., Hasnoot, C. A. G. & Hilbers, C. W. (1982) *Nucleic Acids Res.* **21**, 6981–7000.
10. Roy, S. & Redfield, A. G. (1983) *Biochemistry* **22**, 1386–1390.
11. Roy, S. & Redfield, A. G. (1981) *Nucleic Acids Res.* **9**, 7073–7083.
12. Roy, S., Papastavros, M. Z. & Redfield, A. G. (1982) *Biochemistry* **21**, 6081–6088.
13. Keith, G., Gangloff, J. & Dirheimer, G. (1971) *Biochimie* **53**, 123–125.
14. Sanchez, V., Redfield, A. G., Johnston, P. D. & Tropp, J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5659–5662.
15. Leroy, J. L. & Guéron, M. (1982) *Biophys. J.* **38**, 231–236.
16. Caron, F., Guéron, M., Nguyen Ngoc Quoc Thuy & Herzog, R. F. (1980) *Rev. Phys. Appl.* **15**, 1267–1274.
17. Plateau, P. & Guéron, M. (1982) *J. Am. Chem. Soc.* **104**, 7310–7311.
18. Morris, G. A. & Freeman, R. (1978) *J. Magn. Reson.* **29**, 433–462.
19. Teitelbaum, H. & Englander, S. W. (1975) *J. Mol. Biol.* **92**, 55–78.
20. Kim, S. H., Suddath, F. L., Quigley, G. J., McPherson, A., Sussman, J. L., Wang, A. H. J., Seeman, N. C. & Rich, A. (1974) *Science* **185**, 435–440.
21. Quigley, G. J. & Rich, A. (1976) *Science* **194**, 796–806.