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^{31}P magnetic resonance of tRNA

(molecular structure)

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ABSTRACT **We have observed well-resolved ^{31}P resonances at 65 kG (109 MHz) in solutions of *Escherichia coli* $tRNA^{Glu}$ and yeast $tRNA^{Phe}$. One resolved resonance, identified as the terminal phosphate, titrates with a pK = 6.35. Upon melting the yeast $tRNA^{Phe}$ in 0.1 M NaCl, without Mg^{++}, the resolved peaks broaden and disappear in the vicinity of 35° while the central cluster narrows drastically, shifts slightly, and loses its structure. Addition of Mg^{++} shifts one resolved peak and changes the shape of the cluster. The ^{31}P lines are broader when observed at 65 kG than at 24 kG. The broadening is shown to come from chemical shift anisotropy which is estimated to be about 140 ppm.**

There have been two earlier reports of ^{31}P nuclear magnetic resonance (NMR) of tRNA. In the first of these (1), the discovery of a partially resolved spectrum for purified tRNA was described. Differences between the spectra of yeast $tRNA^{Phe}$ and *Escherichia coli* $tRNA^{Glu}$ were observed. It was suggested that strains were responsible for shifting the ^{31}P resonance. The spectra were obtained in the continuous wave mode at 40 MHz at tRNA concentrations about 1 mM. In the second study (2), the terminal phosphate was observed in an unfractionated tRNA sample but the spectrum of the purified sample did not show any structure, due perhaps to the low concentration used. Upon melting, the main peak narrowed, and an effect of Mg^{++} on melting was observed. In an interesting experiment, the attack of tRNA by pancreatic ribonuclease was followed by the rise and decay of the 2′:3′-cyclic phosphate intermediates 20.4 ppm downfield, and the rise of the 3′-phosphate concentration. The spectra were obtained in the Fourier transform mode at 36 MHz.

In the present paper, we report experiments carried out at higher frequency (109 MHz) in the Fourier transform mode. With the better sensitivity and in some cases better resolution provided by the higher frequency, we have been able to develop the earlier studies and improve earlier characterizations.

EXPERIMENTAL

E. coli $tRNA^{Glu}$ was a gift from Oak Ridge National Laboratory and yeast $tRNA^{Phe}$ was obtained from Boehringer. Sterile buffers and glassware were used. The glassware was leached in 40% nitric acid (2 hr) to remove divalent ions.

For a typical run, 25 mg of tRNA was dissolved in 2 ml of H_2O and dialyzed twice against 0.5 liter of buffer containing 10^{-2} M EDTA for 3 hr, then overnight against 1 liter of buffer containing 10^{-3} M EDTA. The buffer was 0.1 M NaCl, 10^{-2} M cacodylate, with Mg^{2+} where indicated. The tRNA was concentrated by vacuum dialysis to 0.7 ml, the volume of the NMR cell used, and 0.05 ml of D_2O was added for field-locking. The cell was inserted in a 10 mm sample tube, otherwise filled with water. The spectra were taken in the Fourier transform mode on a Brücker HX270 spectrometer, using deuterium lock and proton noise decoupling. The chemical shifts are referenced by substitution to a sample of trimethyl phosphate dissolved in D_2O; therefore, no bulk susceptibility correction need be made. Our shifts are measured relative to an external standard of trimethyl phosphate which is 4.1 ppm downfield with respect to an external standard of H_3PO_4 in a cylindrical capillary along the z axis.

Abbreviation: NMR, nuclear magnetic resonance.

RESULTS AND DISCUSSION

1. Terminal phosphate

Fig. 1 shows spectra of yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Glu}$. The spectra are similar to those obtained earlier (1) but exhibit features impossible to resolve at the time. Most of the phosphate resonances are located in an unresolved cluster whose position is that expected for the 3′:5′-phosphodiesters (3, 4). A number of resonances are scattered on both sides of the cluster. In the spectrum of yeast $tRNA^{Phe}$ the peak at lowest field shown in Fig. 1 at +1 ppm has an area corresponding to $1/76$ of the total, that is, to a single phosphorus. It is located in the region expected for a monophosphate and was identified previously (2) as the terminal phosphate. One notes that this resonance is narrower than the others and this may (but also may not) be due to a greater mobility of this phosphate.

We have performed a titration of yeast $tRNA^{Phe}$ (Fig. 2). Over the pH range explored (5.7–9.5) there is basically no change in the spectrum, except for the low-field line which titrates at pH 6.35, shifting by 3.5 ppm over the entire titration range. There is no other candidate than the terminal phosphate for such a behavior. The titration was carried out by the addition of controlled quantities of 1 M NaOH. This resulted in the appearance of a single extra resonance peak which disappeared after dialysis. We attribute it to phosphate or sugar phosphate, due to degradation of tRNA by alkali before dilution can take place. This peak titrated at pH 7.0. The stronger acidity of the nucleotides (pK = 6.35) has been reported previously (5).

2. The scattered peaks

On both sides of the main cluster, well-resolved peaks are observed, in addition to peaks which merge into the cluster. The shifts from the main cluster are too large to be due only to ring-currents of the bases which could hardly account for even 1 ppm. The magnitude of the shifts induced by hydrogen-bonding of the phosphodiesters is unknown but is ex-

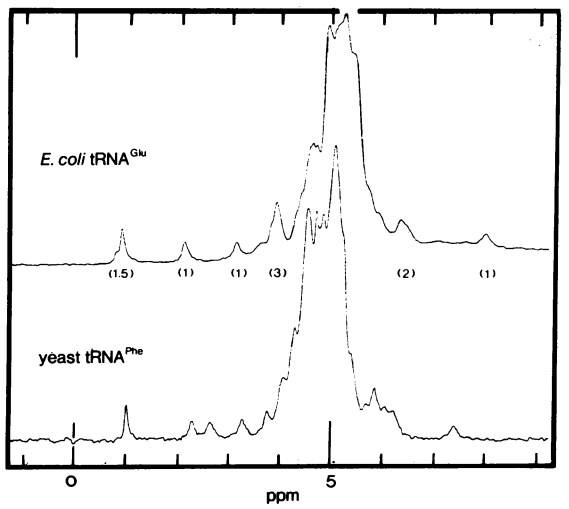

FIG. 1. ^{31}P spectra of tRNA at 109 MHz. The *E. coli* $tRNA^{Glu}$ buffer contained 15 mM Mg^{2+}; the numbers in parentheses give the integrated intensity of the peaks, normalized to a total of 76 ^{31}P nuclei. The buffer for yeast $tRNA^{Phe}$ contained an unknown quantity of Mg^{2+}, probably amounting to about 2.5 Mg^{2+}/tRNA. Free precessions, 8600, repeat time, 4 sec; A/D conversion, 6 bits; memory, 8192 words; broadening, 2 Hz.

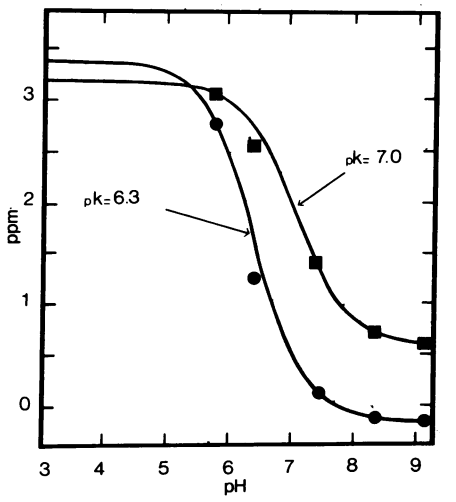

FIG. 2. Titration of the terminal phosphate of yeast $tRNA^{Phe}$, and of sugar phosphate in the same sample. (0.1 M NaCl, 1 nM EDTA, 15 mM Mg^{2+}). The ordinate shows the shifts in ppm from trimethylphosphate.

pected to be much smaller than that due to protonation of the monophosphates, which is about 3 ppm upfield. On this basis hydrogen-bonding of phosphodiesters might explain the less distant upfield-shifted resonances, but not the large shifts observed to high and low fields.

Another possible shift mechanism is straining of the phosphodiesters. For example, whereas the weakly strained 3′:5′-cyclic phosphates are shifted upfield by less than 1 ppm from the phosphodiesters, the highly strained 2′:3′-cyclic phosphates are 20.4 ppm downfield from H_3PO_4 (2, 6, 12). Strains in the tRNA structure might then be the cause of some of the shifted resonances, in particular those to low field. In general the ^{31}P resonances in five-membered cyclic phosphates are found 20–25 ppm to lower fields than in the analogous but less strained six-membered rings (6), while the acyclic compounds are only several ppm downfield from the six-membered rings.

3. Comparison between yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Glu}$

From Fig. 1 it appears that the spectra of these two tRNAs resemble each other, but there are some differences. The general distribution between the cluster and the scattered peaks is similar, and direct correspondence seems possible for some of the latter, e.g., at 1 ppm (terminal phosphate), 2.3, and 3.4 ppm, in the 6 ppm region, and for the isolated peak around 7.8 ppm. The cluster of *E. coli* $tRNA^{Glu}$ seems shifted upfield from that of yeast $tRNA^{Phe}$. This difference is due to the presence of Mg^{2+} in the $tRNA^{Glu}$ sample and its absence in the $tRNA^{Phe}$ since, as discussed below, the addition of Mg^{2+} to $tRNA^{Phe}$ shifts the upfield part of the cluster approx. 0.4 ppm to higher field.

The resemblance between the two spectra is strikingly reinforced by the recent observation of well-resolved lines from a mixture of *E. coli* tRNAs in 15 mM Mg^{2+} at 40 MHz (J. L. Leroy and M. Guéron, private communication).

4. Melting of yeast $tRNA^{Phe}$

The melting of yeast $tRNA^{Phe}$ in 0.1 M NaCl, without Mg^{2+}, is shown in Fig. 3. Starting from 25° let us first consider the changes upon cooling. There is a clear broadening which may be due to the increasing viscosity of the solution. The main cluster changes in a more significant way, with its upfield part increasing in intensity and shifting to higher field. The same type of change is observed upon addition of Mg^{2+} (Fig. 4) and we take it to indicate a tighter ordering of the structure.

Upon heating above 25°, the shape of the main cluster changes, the intensity in the upfield part being transferred to the low-field part. We consider that the high temperature position corresponds to unperturbed phosphodiester groups, whereas the upfield positions reflect perturbations brought about by the secondary and tertiary structure. The nature of these perturbations remains unknown, but moderate strains and consequent electronic rearrangements involved in the formation of double helices is a possible mechanism for the observed upfield shift from 4.6 to 5.2 ppm.

Another feature observed upon heating is the disappearance of the scattered peaks, by broadening and without much displacement. As 35°, from the observed broadening (by $\Delta\nu$ = 20 Hz, half width at half height) of the peaks between 2 and 3 ppm and assuming a reduction of one-half in the total intensity, one derives a lifetime for the unmelted structure responsible for these peaks of $\tau \approx (2\tau\Delta\nu)^{-1} \approx 8$ msec.

Previously, high resolution proton NMR spectra have been reported for yeast $tRNA^{Phe}$ under identical solution conditions (7). In those experiments two or three proton resonances broaden near 38°, indicating that their lifetimes were reduced to <5 msec. Earlier optical measurements by Riesner *et al.* (8) had been interpreted as indicating melting of the tertiary structure near this temperature, while the secondary structure remained intact. This interpretation would be consistent with the proton NMR results (7) and suggests that the ^{31}P broadening is reflecting the same phenomenon. This correlation supports the hypothesis that most of the resolved phosphorous resonances are shifted out of the large cluster by interactions of the tertiary structure. If this is proven they can be used as indicators of the intactness of the tertiary structure.

The terminal phosphate is basically unperturbed during the melting. Its shift is due to pH variations with temperature.

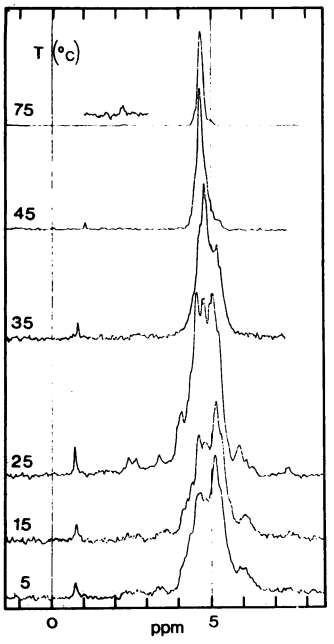

FIG. 3. Melting of yeast $tRNA^{Phe}$ in 0.1 M NaCl, no Mg^{2+}. The shifts were corrected for the shift of the D_2O lock with temperature (0.1245 ppm/°C, upfield).

5. The effect of divalent ions

The effect of divalent ions was studied in the presence of 0.1 M NaCl. Fig. 4 shows the spectrum of yeast $tRNA^{Phe}$ with various quantities of Mg^{2+} added. At the tRNA concentration used the Mg^{2+} should be close to totally bound.

Three features are noticeable:

(*a*) Most prominent is the changed distribution in the main cluster. As Mg^{2+} is added this cluster splits in two distinct parts (see also § 4 in this paper). It is tempting to assign the low-field part to the non-helical regions and the high-field part to the double-helix regions. At high [Mg^{2+}], the intensities are, respectively, 35 and 27 for the high- and low-field parts of the main cluster (from 5.5 to 4 ppm), and 14 for the scattered peaks. In the cloverleaf pattern there are 42 nucleotides in double helices (including the terminal phosphate) and 34 others. Since the splitting of the main cluster is also found in the absence of Mg^{2+} at low temperature (Fig. 3), we consider that it reflects a change in conformation rather than an NMR shift due to Mg^{2+} binding *per se*.

(*b*) Among the well-resolved peaks only one is sensitive to Mg^{2+}, the one located at 2.5 ppm in the absence of Mg^{2+}, which shifts upfield by 0.4 ppm.

(*c*) The terminal phosphate is not shifted at all by Mg^{2+} (but may nevertheless bind the ion).

From its sensitivity to Mg^{++} and temperature, as well as the relative intensity distribution, the central cluster seems to be responding primarily to features of the secondary structure, which are, of course, influenced by the tertiary interactions. Hence it seems likely that the resolved ^{31}P peaks, if assigned, would at first be simpler to interpret.

The effect of Co^{2+} was briefly investigated. At concentrations as low as 0.1 Co^{2+} per tRNA, and in the absence of Mg^{2+}, the line width of the terminal phosphate increased considerably (by 20 Hz). The peak at 2.5 ppm which is shifted by Mg^{2+} was also broadened, as well as the scattered peaks in the upfield side. At 0.2 Co^{2+} per tRNA, the other phosphates broadened also. Note that at the high ionic strength (0.1 M) used here the strong sites for divalent ions are swamped out by salt competition (J. L. Leroy and M. Guéron, private communication), so that it is not surprising that the terminal phosphate is the first to bind cobalt. This is also consistent with the observation that tRNA cannot compete with EDTA for Co^{2+} (or Mg^{2+}): upon addition of either ion, spectral effects occurred only after the EDTA concentration was reached and overcome.

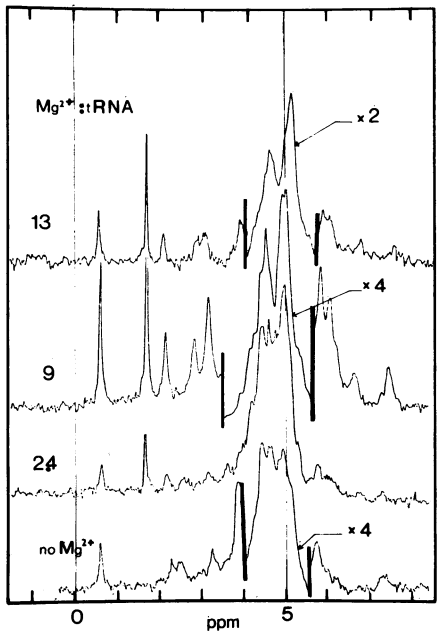

FIG. 4. Effect of Mg^{2+} on the ^{31}P NMR spectrum of yeast $tRNA^{Phe}$ at 25°. The quoted Mg^{2+} concentrations are computed with the assumption that EDTA binds Mg^{2+} before tRNA (see *text*). The sharp peak at 1.6 ppm is spurious sugar phosphate. Note the shift with Mg^{2+} of the peak originally located at 2.5 ppm, and the change in the main cluster.

6. Phosphorus relaxation

The comparison of the spectra obtained here with earlier (1) measurements at low fields shows an increase in linewidth with magnetic field. We have checked this by taking a spectrum of the *E. coli* $tRNA^{Glu}$ sample of Fig. 1 at 40 MHz. The line widths of the scattered peaks were 3 Hz fullwidth at half height, compared to 14 Hz in the present experiments, after correcting for an instrumental broadening of 2 Hz. The field dependence suggests that the relaxing mechanism may be the anisotropic chemical shift, as has been proposed recently in the case of sonicated phospholipid vesicles (9).

This mechanism is in agreement with all the experimental data:

(*a*) It should give rise to a width proportional to the square of field. From the observed width of 14 Hz at 109 MHz one would expect 1.9 Hz at 40 MHz as compared to the observed 3 Hz. It is possible that field-independent mechanisms such as spin-rotation interaction contribute slightly to the low field widths.

(*b*) Assuming this mechanism, and an axial chemical shift tensor, one can compute the chemical shift anisotropy $\delta \equiv \delta\| - \delta\perp$ by the formula (p. 315 of ref. 10):

$$\frac{1}{T_2} = \frac{1}{20}\omega^2\delta^2[4\tau_c + 3\tau_c/(1+\omega^2\tau_c^2)]$$

where τ_c is the rotational correlation time,

$$\tau_c = 4\pi\eta a^3/kT$$

With a Stokes radius $a = 29$ Å (11), we obtain $\delta \cong 136$ ppm, a reasonable value for phosphates.

(*c*) With this value of δ, we can compute T_1 by:

$$\frac{1}{T_1} = \frac{3}{10}\omega^2\delta^2\frac{\tau_c}{1+\omega^2\tau_c^2}$$

which gives $T_1 = 4.5$ sec. From approximate direct observations we estimated T_1 to be between 2 and 5 sec.

The determination of the relaxation mechanism leads us to considerations of resolution and sensitivity. First, it is clear that resolution diminishes at high field since the width due to chemical shift anisotropy increases as ω^2 whereas the chemical shift splittings go as ω only (9). Second, the sensitivity (signal-to-noise ratio obtainable in a given time) is (p. 83 of ref. 10)

$$(S/N)_{\text{power}} \propto \omega^3\, T_2/T_1,$$

all instrumental factors being equal. The T_2/T_1 dependence is quite general. In continuous wave it is easily visualized from the steady state solution of the Bloch equations at optimum radio frequency field, while in Fourier transform it stems basically from the fact that the free precession lasts for a time T_2 and is repeated after T_1. Let us compare the case of protons relaxed by a dipolar interaction and of phosphorus relaxed by chemical shift anisotropy, assuming $\omega\tau_c \gg 1$. For the dipolar interaction T_2 is field-independent and T_1 increases as ω^2. In the case of chemical shift anisotropy, T_2 decreases as ω^{-2} and T_1 remains constant. In both cases T_2/T_1 decreases as ω^{-2}, so that the sensitivity varies as ω^1. Thus, the sensitivity increases with field in the same way for protons and phosphorus.

7. Other remarks

(*a*) In some instances we observed one or two resonances downfield at a position corresponding to the 2′:3′-cyclic phosphate (−16.25 ppm). Since 2′:3′ phosphates are an intermediate in the hydrolysis of tRNA, we assume that this is evidence of partial degradation. The signal could not be dialyzed away and is therefore ascribed to cuts in tRNA. It corresponded typically to one or two cuts per tRNA and did not evolve in time; the cuts may have been introduced during the isolation procedure.

(*b*) While none of the phosphodiester resonances has as yet been identified, examination of the Cambridge model reveals a number of critically located phosphates, which could be strained and/or hydrogen bonded. We feel that the ^{31}P resonances will be very helpful in following the solution structure of tRNA molecules.

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