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A Comparison of the Fluorescence of the Y Base of Yeast $tRNA^{Phe}$ in Solution and in Crystals†

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ABSTRACT: The fluorescence properties of the Y base of yeast $tRNA^{Phe}$ are known to be quite sensitive to the environment. The fluorescence lifetime of the Y base in yeast $tRNA^{Phe}$ is identical in orthorhombic crystals and in the mother liquor from which these crystals are grown. It is 10% higher than the lifetime observed in dilute solutions of tRNA. This small change is a solvent effect due to isopropyl alcohol in the crystallization medium. Isopropyl alcohol does not change the accessibility of the chromophore of the Y base as measured by iodide quenching rates in solution. The accessibility in intact $tRNA^{Phe}$ is much less than in a ribonuclease digest. Thus, within the limits of the sensitivity of the method, the Y chromophore occupies the same environment in solution and in the crystal and it must be at least partially buried.

High-resolution X-ray crystallography is the most powerful tool for the elucidation of the three-dimensional structure of proteins and nucleic acids. There are always lingering doubts, however, whether the structure determined in the crystal is the same as the structure in solution. This is especially true when one considers the relatively weak interactions that stabilize the tertiary structure of biopolymers. This uncertainty is particularly significant for tRNA. The solution structure as measured by circular dichroism (CD) is quite variable, depending on specific solvent conditions (Prinz et al., 1974), which may indicate that structural changes occur during in vivo protein synthesis. Also, at least eight different crystal forms have been observed for yeast $tRNA^{Phe}$, some containing up to 80% solvent (Cramer et al., 1974). X-Ray diffraction studies on two crystal forms have yielded the three-dimensional structure of this tRNA (Kim et al., 1974; Robertus et al., 1974). Low field nuclear magnetic resonance NMR (Kearns and Shulman, 1974) and singlet-singlet energy transfer measurements (Yang and Soll, 1974) are consistent with a tRNA solution structure similar to that found in crystals. However, these mea-

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Table I: Fluorescence Characteristics of Yeast tRNA^{Phe} in Solution and in Orthorhombic Crystals.

Expt	Sample	$\lambda_{ex.\ max}$ (nm)	$\lambda_{em.\ max}$ (nm)	τ(nsec)
1	Mother liquor	320	422	8.4
	Crystal	332	445	8.4
2	Mother liquor	315	437	8.2
	Crystal	325	430	8.2
3	Mother liquor	320	450	
	Crystal	323	440	
Av[a]	Mother liquor	318	436	8.3
Av[a]	Crystal	327	438	8.3
Av[b]	Dilute solution	318	428	7.5

[a] Average values from experiments 1–3. [b] Average of several measurements of 0.1 mg/ml of tRNA in same buffer without isopropyl alcohol.

surements can be performed easily only on solutions and not on actual crystals. Their ability to yield detailed three-dimensional information is quite limited.

The occurrence of the environmentally sensitive fluorescent Y base (Nakanishi et al., 1970; Beardsley et al., 1970) in tRNA^{Phe} offers the opportunity to examine in considerable detail one specific structural region, the anticodon loop. The following measurements of Y base fluorescence both in solution and in crystals, have two goals: to demonstrate the feasibility of obtaining quantitative fluorescence data from very small volumes of crystalline samples and to compare the structure of the tRNA^{Phe} in solution and in crystals.

Experimental Section

Crystals of yeast tRNA^{Phe} were prepared by the procedure of Kim et al. (1971). Droplets of tRNA solutions were placed in depressions in a glass plate. The solutions contained tRNA, 12 mg/ml; $MgCl_2$, 10 m*M*; cacodylate buffer, 10 m*M*, pH 6.0; spermine-HCl, 1 m*M*; the volume of solution was 50 μl/drop. The high concentration of tRNA was deliberate to produce many small crystals. The glass plate was placed in a sealed plastic box and equilibrated through the vapor phase with 25 ml of 8% v/v isopropyl alcohol in H_2O at 4°. Crystals were allowed to grow for 2 weeks under these conditions. The equilibrating buffer was then changed to 30% v/v isopropyl alcohol in H_2O to stabilize these crystals. Under these conditions two crystal forms were observed. The first to form are elongated prismatic crystals of the hexagonal system ($P6_122$). This form is the first to appear probably because of easy nucleation, but under these conditions the hexagonal form is less stable than the orthorhombic. As the hexagonal crystals dissolve, radiating clusters of bladed crystals of the orthorhombic system ($P2_122_1$) appear. The entire crystallization procedure was carried out at 4° but after equilibration with 30% isopropyl alcohol, the orthorhombic crystals are stable at 25°, the temperature used for the fluorescence measurements. In a typical crystallization, the hexagonal form began to appear after approximately 24 hr, and the orthorhombic form became dominant after the fifth day. The typical size of the crystals are as follows: hexagonal, ~100 μ, length × 25 μ in diameter; orthorhombic, 100 μ × 30 μ × 5 μ.

Fluorescence samples were prepared by the following procedure. Fused quartz tubing, spectrosil grade, was drawn into capillaries approximately 1 mm in diameter and 10 cm in length. These capillaries were washed with concentrated HNO_3 to remove fluorescent impurities before use. A small sample of supernatant was drawn off and sealed in the capillary. The remaining supernatant containing the crystals was collected in another capillary and after the crystals settled the excess supernatant was removed leaving a small droplet consisting of a surry of crystals with very little excess mother liquor. In addition, 10-μl samples of supernatant were collected to determine the tRNA concentration in the mother liquor. The capillaries were then mounted in the center of standard 1-cm^2 fluorescence cuvets using plastacene clay. Both the supernatant and crystal samples were approximately 3 mm in length giving a sample volume of 2.5 μl.

Solution studies were performed in the same buffer as for crystallization (without isopropyl alcohol) using 0.1 mg/ml of tRNA in standard 1-cm^2 fluorescence cuvets. Potassium iodide or isopropyl alcohol was added as specified in individual experiments.

Fluorescence spectra were obtained on a Perkin Elmer MPF-2A spectrofluorimeter. Fluorescence lifetime measurements were performed on a single photon counting apparatus described previously (Tao, 1969).

Results and Discussion

The following results on tRNA crystals were all obtained from orthorhombic crystals. These small crystals were prepared in parallel with other identical samples which were subsequently allowed to grow large and then examined by X-ray diffraction. This showed definitively that the crystals were of the same orthorhombic form from which the tertiary structure has recently been determined (Kim et al., 1974). Crystals were closely packed into a capillary as described above. Several attempts were made to perform the experiments on the hexagonal ($P6_122$) form, but the crystals were too unstable, and would dissolve in the capillary before measurements could be made. The stability of the orthorhombic crystals was verified by microscopic examination of the capillary between crossed polarizers before and after the experiments. The observed birefringence indicated that the sample was still crystalline at the end of the experiment.

Spectroscopic data obtained from samples mounted in capillaries are summarized in Table I. The main feature of the results is that the Y base fluorescence is very similar in the crystal, mother liquor, and the dilute tRNA solutions. The excitation spectra show a consistent red shift for the crystal sample relative to solution. Inspection of the spectra in Figure 1a indicates that this apparent shift is due to an inner filter effect from the high concentration of tRNA in the sample. The calculated absorbance of the crystal sample at 320 nm in a 0.8-mm capillary is approximately 5, which is 1000 times higher than the mother liquor at this wavelength (see Table II). The absence of excitation in the region from 240 to 300 nm indicates that virtually all the radiation in this region is absorbed by the tRNA. Since the crystals were fairly densely packed the total sample absorbance and the resultant red shift should be independent of crystal size. The emission wavelengths were observed to be quite variable. Two examples are illustrated in Figure 1b. The observed variations do not correlate well with sample type and all appear to be the results of a weak signal with very broad maximum. No systematic difference in emission is observed between the crystal and mother liquor samples. Measurements were also made of the fluorescence lifetime

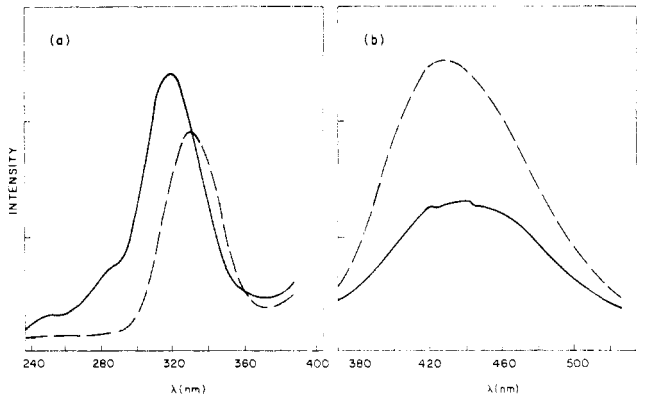

FIGURE 1: (a) Uncorrected excitation spectra of tRNA crystals (- - -) and mother liquor (—). (b) Uncorrected emission spectra of mother liquor (—) and dilute tRNA (0.1 mg/ml) in buffer without isopropyl alcohol (- - -). Emission and excitation intensities do not correspond because they were obtained on different samples.

of the Y base in these samples. The results are summarized in Table I. The lifetimes were found to be identical for samples of crystals and their mother liquor. These lifetimes were, however, consistently longer than dilute tRNA in crystallization buffer without isopropyl alcohol.

In order to evaluate these results it is necessary to prove that the fluorescence of the crystal sample is from the crystals and not from the mother liquor in which they are densely suspended. The crystals contain approximately 70% H_2O and have a density of 1.17 g/ml (Kim, unpublished result). This leads to tRNA concentrations in the crystal of approximately 350 mg/ml. The measured concentration in the mother liquor was found to be 0.5 mg/ml. Since the fluorescence lifetimes are the same, the observed fluorescence intensity should be proportional to the amount of exciting light absorbed. The absorbance can be calculated from the known concentration, path length, and extinction coefficients as shown in Table II. From the absorbances one can predict that the relative fluorescence intensity of crystals and supernatant should be only 40:1 although the relative tRNA concentration is almost 1000:1. The observed intensity ratio for the 1-mm capillaries of 30:1 agrees well with this calculation verifying that the fluorescence of the crystal samples does not come from the mother liquor associated with the crystals. The difference in predicted and observed values probably arises from the fact that the crystals are not packed solidly.

Much evidence has accumulated that the fluorescence of the Y base in $tRNA^{Phe}$ is a sensitive indicator of the local structure (Beardsley et al., 1970; Eisinger et al., 1970; Maelicke et al., 1973; Zimmerman et al., 1970; Robison and Zimmerman, 1971; Blumberg et al., 1974). Thus, the preceding results indicate that the Y chromophore (the aromatic portion of the Y base) is in the same environment in both the crystal and supernatant. However, the 10% discrepancy in lifetimes between these samples and the dilute solution samples needs to be explained. Prinz et al. (1974) have studied the CD of $tRNA^{Phe}$ under different crystallization conditions. Their results indicate that the secondary structure is partially disrupted when compared to native $tRNA^{Phe}$. Beardsley et al. (1970) showed that disruption of $tRNA^{Phe}$ structure by Mg^{2+} removal or nuclease digestion led to a decrease in Y base fluorescence. This is opposite to the effect we observe with isopropyl alcohol. Therefore, one possibility is that isopropyl alcohol is causing an increase in structure near the Y base. An alternative is a simple solvent

Table II: Calculated Absorbance and Fluorescence Properties of tRNA Samples.

	Crystal	Mother liquor
tRNA concentration (mg/ml)	350	0.5
Absorbance at 260 nm (A_{260}/mm)[a]	700	1
Absorbance at 320 nm (A_{320}/mm)[b]	7	0.01
Fraction of light absorbed at 320 nm (%)	100	2.5
Calculated relative fluorescence intensity	40	1
Observed relative fluorescence intensity	30	1

[a] Calculated for 1-mm path length using ϵ_{260} (tRNA) = 1.3×10^5. [b] Calculated for 1-mm path length from the experimentally measured ϵ_{320} (tRNA) = 1.3×10^3.

Table III: Effect of Isopropyl Alcohol and Nuclease Digestion on the Fluorescence and Iodide Quenching of $tRNA^{Phe}$ Solutions.

Sample	F_0[a]	τ_0 calcd[b] (nsec)	τ_0 obsd (nsec)	$k_q \times 10^{-9}$ (M^{-1} sec^{-1})
$tRNA^{Phe}$	100	7.5	7.5	0.18
$tRNA^{Phe}$ + 30% isopropyl alcohol	107	8.1	8.3	0.18
$tRNA^{Phe}$ + RNase	27	2.0		0.95
$tRNA^{Phe}$ + RNase + 30% isopropyl alcohol	37	2.8		0.50

[a] Relative fluorescence intensity before quenching. [b] Calculated from F_0 using 7.5 nsec for the fluorescence lifetime of Y base in $tRNA^{Phe}$.

effect on the fluorescence of the Y chromophore. Beardsley et al. (1970) found that the fluorescence of the isolated Y base is markedly enhanced by nonpolar solvents. To determine which of these mechanisms is responsible for the observed lifetime differences, iodide quenching experiments were performed.

Heavy atom quenching of fluorescent species in solution has often been used to give an indication of the degree to which a given chromophore is exposed to the solvent (Lehrer, 1971). The mathematical interpretation of fluorescence quenching was first derived by Stern and Volmer (1919). Purely collisional quenching obeys the following equation:

$$F_0/F = 1 + k_q\tau_0[Q]$$

where F_0 and τ_0 are the intensity and lifetime of the fluorophore before quencher is added. F is the observed intensity at a given quencher concentration, [Q]; k_q is rate constant for collision of quencher with excited fluorophore. Thus k_q should be a measure of the accessibility of the fluorophore.

Recent studies by Lakowicz and Weber (1973) indicate the effectiveness of iodide ion as a quencher for studies of the exposure of chromophores in biopolymers. Therefore, the effect of varying iodide concentrations on the fluorescence of the Y base of $tRNA^{Phe}$ was examined. These results are summarized in Table III. Four samples were employed: intact $tRNA^{Phe}$ and $tRNA^{Phe}$ digested by the addition of 5 μg/ml of bovine pancreatic RNase were examined in both aqueous buffer and in the presence of 30% isopropyl

alcohol as used in crystallization. The RNase digestion produces fragments of the tRNA including a hexanucleotide containing the Y base (RajBhandary et al., 1968).

All samples gave linear Stern–Volmer plots over a quencher concentration range of 0–0.3 M iodide ion. The quenching constants shown in Table III were calculated from the slopes of $(F_0/F) - 1$ vs. $\tau_0[I^-]$. Iodide quenching was also examined by direct determination of fluorescence decay times for intact tRNA^{Phe} in the presence and absence of I^-. The fractional change in lifetime was in good agreement with changes in static fluorescence. This shows that the observed quenching is dominated by pure collisional quenching. No corrections in k_q values were made for viscosity changes due to isopropyl alcohol addition. There is considerable evidence that changes in viscosity of a solution have a much smaller effect on the diffusion rate of small molecules than would be predicted from the Einstein diffusion equations (Ware, 1962; Jordan et al., 1956).

The iodide quenching constant for intact tRNA^{Phe} is unaffected by isopropyl alcohol. This clearly indicates that addition of isopropyl alcohol increases the fluorescence efficiency without changing the degree of exposure of the Y chromophore to the solvent. We conclude that, within the sensitivity of fluorescence measurements, the environment of the Y chromophore is the same in dilute solutions of tRNA^{Phe} and in orthorhombic crystals of tRNA^{Phe}.

The iodide quenching constant of RNase fragments is much larger than in intact tRNA^{Phe}. This indicates that the Y chromophore is much more exposed to solvent in these fragments. It appears that the degree of exposure depends on more than just the nearest neighbor nucleotides around the Y base. Addition of isopropyl alcohol actually decreases the accessibility of the Y chromophore in RNase fragments.

Previous observations suggested that the anticodon loop of tRNA^{Phe} has a highly ordered structure with the Y chromophore well protected from the solvent. Beardsley et al. (1970) observed that changing the solvent from water to D_2O caused large fluorescence changes in the Y base. No change, however, was observed when intact tRNA^{Phe} was dissolved in a D_2O buffer (Beardsley et al., 1970). Our present results demonstrate a very large fluorescence change and a large change in quenching constant when alcohol is added to a solution of tRNA fragment. However, in the intact tRNA the fluorescence change due to the alcohol is very small. The iodide quenching data also indicate a fivefold decrease in the accessibility of the Y chromophore to the solvent in intact tRNA when compared to the hexanucleotide fragment from the RNase digestion. These data strongly support the earlier conclusion that the anticodon region in intact tRNA has a highly ordered structure protecting the Y chromophore from the solvent. This structure does not exist for the hexanucleotide containing the Y base, therefore, the conformation of the anticodon loop must be stabilized by other regions of tRNA such as the anticodon stem.

These conclusions about the environment of the Y chromophore are consistent with the X-ray cystallographic results (see Figure 2). At 3-Å resolution, the crystal structure of yeast tRNA^{Phe} in orthorhombic form (Kim et al., 1974) shows that the Y chromophore is sandwiched between two adjacent bases, A36 and A38, providing good stacking interaction to stabilize the conformation near the Y base. Besides, the circumference of the Y chromophore is protected by two methyl groups and the large side chain of the Y base, thus shielding most of the aromatic portion of the Y

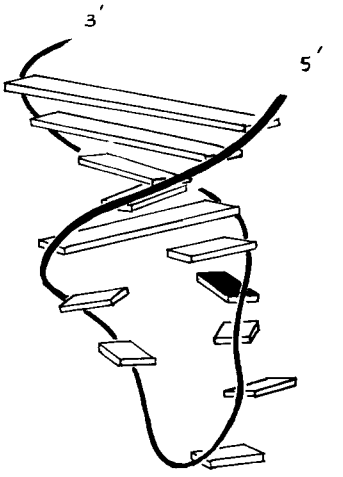

FIGURE 2: Schematic drawing of the anticodon stem and the loop of yeast tRNA^{Phe} in orthorhombic crystal from X-ray crystallographic studies (Kim et al., 1974). The long slabs indicate base pairs and the short ones simple bases in the loop. The Y base is the black slab which is a part of five stacked bases.

base. Figure 2 is a schematic drawing of the anticodon stem and loop. Five bases on the 3′ side of the loop are stacked. It is likely that any conformational change among these five nucleotides will be felt by the Y base, thus changing the fluorescence lifetime and the accessibility to iodide ion quenching.

One of the purposes for undertaking these experiments was to test the effectiveness of existing fluorescence techniques and instrumentation for small samples of crystalline biopolymers. The sample volume for these experiments was only 2.5 μl which corresponds to approximately 1 μg of tRNA in the mother liquor sample (approximately 20 ng of the Y base chromophore). Signal intensity, however, was sufficient for quantitative study which illustrates the great sensitivity of fluorescence techniques. The crystalline samples had such a high optical density that the static fluorescence is clearly no longer proportional to chromophore concentration. Thus fluorescence decay methods were much more reliable than static techniques.

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