*SPIN-LABELED TRANSFER RNA**

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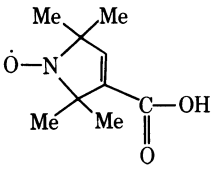
Abstract.—The α-amino group of valyl-tRNA has been combined chemically with a nitroxide spin label through an amide linkage. This spin-labeled aminoacyl-tRNA has an electron paramagnetic resonance spectrum which reflects the mobility of the aminoacyl end of the tRNA molecule. The rate of tumbling of the spin label has been measured as a function of temperature and ionic strength. An abrupt transition in the nature of the motion occurs at a temperature which is sensitive to the ionic strength of the medium. This change in behavior of the aminoacylated spin label occurs at the same temperature as the midpoint of the optical density melting curves in dilute salt solutions. However, when denaturing agents such as dimethylsulfoxide or urea are added to the solution, the spin melting temperature is lower than the melting temperature as measured by the change in optical density. This suggests that under these conditions the local region of the aminoacyl end of the molecule denatures before the rest of the molecule is disrupted.

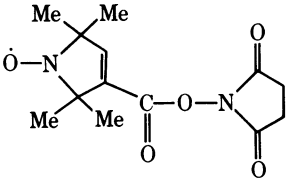
Transfer RNA (tRNA) molecules have been studied by a variety of techniques in an attempt to learn something of their structural organization. The nucleotide sequences of a number of purified tRNA's have clearly disclosed common features which suggest that these molecules may have a common secondary structure. This is of great interest, since tRNA plays a central role with the ribosome in the translation of genetic information into proteins. Most physicochemical measurements such as changes in absorbance, viscosity, or circular dichroism are integral measurements that represent an average over the entire 70–80 nucleotides of the molecule. However, it is desirable to supplement these studies with measurements directed toward a particular portion of this macromolecule. This can be done effectively by using electron paramagnetic resonance (EPR) labels which can be attached to selected portions of the molecule with the techniques developed by McConnell and his colleagues.[1] By measuring the relaxation or tumbling of the attached spin label, one can learn something about the mobility of one portion of a large molecule. We have applied this technique to a study of aminoacylated tRNA by covalently linking a nitroxide spin label to the α-amino group of valyl tRNA (Val-tRNA) via an amide bond. We are thus using a naturally occurring linkage which is not likely to disturb the normal configuration of the molecule. These studies show a sharp discontinuity in the motion of the spin label which occurs at a temperature that is a function of the ionic environment. The changes in spin mobility reflecting the behavior of a portion of the molecule are compared with optical density changes that reflect the entire molecule. Under appropriate denaturing conditions we have been able to demonstrate a considerable difference

between the temperature at which changes occur in spin motion and the melting temperature as determined by changes in the ultraviolet absorbance. We conclude from these studies that it is possible to carry out a structural rearrangement in part of the tRNA molecule while the remainder is relatively unperturbed.

Materials and Methods.—*E. coli* B tRNA and uniformly labeled C^{14}-L-valine (specific activity, 10 mc/mM) were obtained from Schwarz BioResearch. 2,2,5,5-Tetramethylpyrroline-3-carboxyamide (Frinton Laboratories, New Jersey) was converted to the nitroxide carboxylic acid (I) as described by Rozantzev and Krinitzkaya.[2] 530 mg of I and 331 mg of N-hydroxysuccinimide were suspended in 6 ml of ethyl acetate and cooled in an ice bath; 595 mg of dicyclohexylcarbodiimide in 2 ml of ethyl acetate was added with stirring. After 15 min, the mixture was taken out of the ice bath and stirred at room temperature for a further 2 hr. The mixture was filtered, and the solid was washed with ethyl acetate until colorless. The combined filtrate and washings were evaporated to dryness under reduced pressure. The yellow crystalline residue, recrystallized from methanol, gave 635 mg (78%) of *2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxylic acid N-hydroxysuccinimide ester* (II) as yellow plates. Melting point 185–187°C. *Found:* C, 55.5%; H, 6.26%; N, 9.93%. *Calculated for* $C_{13}H_{17}N_2O_5$: C, 55.5%; H, 6.10%; N. 9.96%.



I



II

^{14}C-Val-tRNA was prepared from unfractionated *E. coli* B tRNA by standard techniques with a crude, dialyzed 100,000 × *g* supernatant enzyme preparation from *E. coli* A19. A charging level of 2.48 mμmoles Val/mg tRNA was obtained. A solution of Val-tRNA (5 mg/ml) in 0.05 *M* phosphate buffer (pH 6.8) was mixed with half its volume of a solution of II in acetonitrile (14 mg/ml), and the mixture was kept at 37° for 5 hr. The RNA was then precipitated with ethanol and freed from excess spin label by repeated reprecipitation. A sample of uncharged tRNA, carried through this spin-labeling procedure and re-isolated, retained 80–90% of its original valine acceptor activity. The spin-labeled aminoacyl tRNA was purified by chromatography on hydroxyapatite,[3] and appropriate fractions (as determined by an acid-insoluble radioactivity assay) were pooled, dialyzed, and concentrated. The stock solution of *R*-Val-tRNA used to collect the data of Table 1 contained 11.0 mg/ml of tRNA (*R* is the spin label). Further details of these procedures will be given in reference 5.

The number of nitroxide residues attached to the amino group of Val-tRNA was determined by measuring the per cent of the total acid-insoluble radioactivity stable to treatment with cupric ions.[4] This number was then compared with the total number of nitroxide residues/mole tRNA as determined by complete digestion of the spin-labeled Val-tRNA with alkali and comparison of the EPR signal strength with that given by a standard solution of I.[5] The spin label (I) is stable to this treatment.[2] Some reaction of the acylating agent (II) occurs with uncharged tRNA,[5] but the interference by this "background" labeling was minimized by including the chromatographic purification step. The specimen of *R*-Val-tRNA used in these studies had at least 90% of the total nitroxide molecules bound to the α-amino group of the aminoacyl-tRNA. Most EPR spectra were taken with a Varian E-3, 9.5-GHz spectrometer equipped with a Varian gas-flow temperature-control unit. Samples for study of melting behavior by EPR were prepared by sealing the final solution into a Pyrex capillary tube. By following this procedure, as little as 25-μl total volume can be used for the determination of a melting curve.

To take spectra, the capillary tubes were placed in a 4-mm diameter quartz sample tube containing a 0.005-inch copper constantan thermocouple, and the tube was then filled with xylene over the length of the capillary to ensure thermal contact. Temperatures were measured with the loaded quartz tube in place inside the variable-temperature Dewar mounted in the EPR cavity and are accurate to better than $\pm 1°$. Over the temperature ranges used, the changes in the label's rotational freedom with temperature are reversible and hydrolysis is not a significant factor.

Optical melting curves were measured by diluting aliquots of a concentrated stock solution of unfractionated *E. coli* tRNA into the desired solvent (aqueous dimethylsulfoxide or various concentrations of urea in 1 mM acetate buffer (pH 5), 1 mM EDTA). Solvent expansion in the aqueous dimethylsulfoxide (Me_2SO) system was corrected for by measuring the absorption changes of a solution of cytosine in the appropriate aqueous Me_2SO solution. All optical density measurements were made at 280 $m\mu$ in order to reduce the solvent absorbance of DMSO. Solvent expansion in the aqueous urea solutions was insignificant. Melting temperatures (T_{OD}) were taken as the midpoint of the rise in absorbance when the full curve is visualized.

Results.—We are using a spin-labeling reagent which has a high degree of specificity for α-NH_2 groups,[6] and therefore it is possible to charge unfractionated tRNA with one amino acid, such as valine, and then add the spin label. This allows us to make measurements on Val-tRNA and exclude other tRNA species. When the label is freely tumbling in solution, its spectrum consists of three narrow lines of equal intensity (Fig. 1*a*). Because of the anisotropy in the g tensor and hyperfine tensor, this spectrum is very sensitive to the rate and nature of the motion of the spin label. As the rate of tumbling slows from that in free solution, the three lines broaden unequally and the spectrum becomes asymmetric. A slightly broadened spectrum, such as that in Figure 1*b*, is characteristic of "partial" or "weak" immobilization of the spin label. From such a spectrum the rotational correlation time (τ) can be calculated. Tau is related to the time required for the label to rotate an average of 40° and is a measure of the rotational freedom of the label. A large value of τ indicates slow rotation, while rapid tumbling is associated with low values of τ. Changes of macromolecular configuration influencing the surroundings of a covalently attached spin label are reflected in the label's degree of immobilization, which can be given a quantitative expression in terms of τ.[1]

EPR spectra of the spin-labeled tRNA preparations were found to be characteristic of partially immobilized spins. In all cases the absorption lines became narrower (signal heights increased) with increasing temperature, indicating an increased freedom of rotation for the label (Fig. 1*b*, *c*). Under conditions of partial immobilization, the Kivelson formalism[7] can be used to calculate τ, as demonstrated by Stone *et al.*[8] Independent values of τ can be obtained from the terms linear and quadratic in the nitrogen nuclear spin quantum number. Although in our ex-

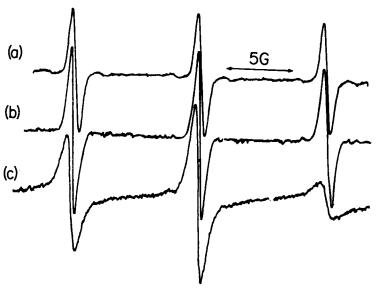


FIG. 1.—EPR spectra of (*a*) spin label I (2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxylic acid) in free aqueous solution; (*b*) *R*-Val-tRNA in 10^{-3} *M* Na acetate, 10^{-3} *M* EDTA, pH 5, $T = 67°$; (*c*) *R*-Val-tRNA in the same buffer as (*b*) at $T = 4°$.

periments these two values do not agree in general, their temperature variation is identical. We shall discuss only the value of τ derived from the term quadratic in the nuclear spin quantum number. We have found that the value of τ from the linear term is sensitive to microwave power, whereas that from the quadratic term is essentially unaffected by microwave power far higher than any used in this study. This practice means that the absolute values of τ and activation energies derived from the temperature variations of τ are uncertain to approximately the 15 per cent or smaller difference between the values derived from the linear and quadratic terms. However, our primary interest is in changes in τ with temperature and with environment and in changes of activation energies; hence, this raises no difficulties.

Figure 2 shows log τ of *R*-Val-tRNA as a function of inverse temperature. At high ionic strength, a single straight line is observed. However, at lower ionic strength, there are two straight-line segments with different slopes which correspond to different activation energies. It should be emphasized that these line segments do not join smoothly, but rather the exponential relation at lower temperatures gives way abruptly at a definite temperature (T_{sp}) to a different exponential relation over higher temperatures. At low ionic strength this is usually associated with a slight discontinuity in the value of τ as well as in slope. For this reason, the results are not considered to be a consequence of two independent modes of motion. If this were the case, then $1/\tau = 1/\tau_1 + 1/\tau_2$, $\tau_i = \tau_i^0 e^{\Delta i/TR}$ and $\ln(\tau)$ would be a smooth function of inverse temperature. Instead, the results are interpreted as arising from one type of motion below T_{sp} and another above. For this case $\tau_1 = \tau_2^0 e^{\Delta_1/RT}$, $T < T_{sp}$; $\tau_2 = \tau_2^0 e^{\Delta_2/RT}$, $T > T_{sp}$. Each of these processes is characterized by its individual activation energy Δ_1 or Δ_2. The melting behavior of *R*-Val-tRNA has been studied by EPR over an extended range of K^+ concentrations, at two different pH values, and in the presence of Mg^{++}. Table 1 gives the values of T_{sp} and the activation energies Δ_1 and Δ_2. The values given are generally the result of two or more runs on each of two samples. Although the activation energies do not change markedly as the ionic environment varies, the value of T_{sp} increases with increasing potassium ion concentration. The values of T_{sp} obtained have uncertainties of about $\pm 2°$.

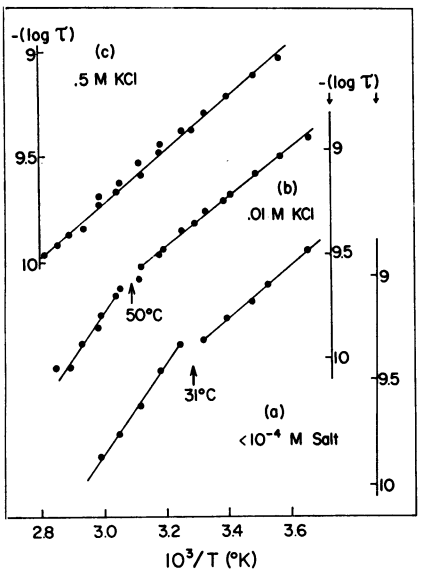


FIG. 2.—Log τ versus inverse absolute temperature of *R*-Val-tRNA at three different concentrations of salt: (*a*) less than 10^{-4} *M* salt; (*b*) 0.01 *M* Na acetate, pH 5, 0.01 *M* KCl; (*c*) 0.01 *M* Tris, pH 7.4, 0.5 *M* KCl. The data plotted in (*b*) and (c) are the results of two independent melting experiments.

Values for the midpoints of the melting curves of unfractionated tRNA as

TABLE 1. *Spin melting and optical density melting data.*

Salt	pH	T_{sp}(°C)	Δ_1	Δ_2	T_{OD}(°C)
Distilled H_2O	7	30°	5.8	10.2	
10^{-3} *M* NaAc, 10^{-3} *M* EDTA	5	36°	5.5	9.8	37.5°
0.01 *M* NaAc, 0.01 *M* KCl	5	50°	5.3	10.0	49.5°
0.01 *M* Tris, 0.01 *M* KCl	7.4	47°	6.0	9.7	
0.01 *M* Tris, 0.01 *M* $MgCl_2$	7.4	70°	5.4	(11.7)	75°*
0.01 *M* Tris, 0.5 *M* KCl	7.4	—	5.7	—	

* Value obtained in 0.01 *M* Tris (pH 7.2), 0.01 *M* MgAc, 0.2 *M* NaCl.[9]

measured by increase of optical absorption at 280 mμ (T_{OD}) are also contained in Table 1. Comparison of the values of T_{sp} with the T_{OD} values obtained under identical conditions shows a striking parallel between the two types of measurement. Although unfractionated tRNA was used for the OD measurements, there is good agreement between the melting point of purified $tRNA^{Val}$ and that obtained with unfractionated material.[9] The agreement between the optical-density melting and the spin melting temperatures suggests that they both reflect the same process, namely, a temperature-dependent structural transformation. In addition, the sharpness of the break in the curves in Figure 2 suggests that the aminoacyl end of tRNA is not freely moving, but that it participates in the ordering present at low temperatures. The mode of motion which is characteristic of the low-temperature conformation gives way to a new mode at a well-defined stage in the process of thermal denaturation.

The existence of two spin mobility states as a function of temperature in a spin-labeled macromolecule is not a general property. In experiments reported elsewhere,[5] we have shown that spin-labeled poly-L-lysine in a random-coil form and tRNA reacted with the spin-labeling reagent II *after* removal of attached amino acids by incubation at high pH[5] have only one exponential dependence of τ over all temperatures, with an activation energy similar to that seen in the low-temperature form of *R*-Val-tRNA. This is precisely what we would expect for random-coil polylysine, which has neither well-defined secondary or tertiary structure nor a unique conformation of the lysine side chains, and for tRNA randomly labeled in nonhelical regions. Moreover, since the curves for spin-labeled, stripped tRNA show no break, the presence of one can only be attributed to those nitroxide molecules attached via the amino acid to tRNA. The break temperature could be masked by a large excess of nonspecifically attached spin labels, but if observable, its value cannot be distorted by them. Spin label experiments with ribonuclease[10] exhibit a phenomenon similar to that described here in that there is both a low-temperature and a high-temperature mobility. This would be expected in a molecule which undergoes a substantial structural transition on denaturation.

The process which we are observing in dilute salt solutions of tRNA is one in which denaturation of the molecule is accompanied by an abrupt change in the type of rotatory motion of the spin label attached to the amino acid. We

do not know the detailed nature of the origin of molecular motion in the denatured state, but it is characterized by a higher activation energy than that seen in the low-temperature form, as shown in Table 1. In order to learn something about the nature of the forces restricting the motion of the aminoacyl end of tRNA, EPR spectra were obtained in the presence of various concentrations of denaturing agents, and a comparison was made between the optical density and spin denaturation. As noted above, these denaturation temperatures are the same in dilute salt solutions. Figure 3 shows the effect of dimethylsulfoxide on the thermal melting of *E. coli* tRNA as measured both by changes in the absorbance at 280 mμ and as determined by the variation in the rate of tumbling of the spin label. Me_2SO is a polar molecule known to destabilize stacking interactions and to change the dielectric constant of the solution. As can be seen in Figure 3*a*, only small changes in the absorbance melting curve occur upon addition of Me_2SO up to a concentration of 50 per

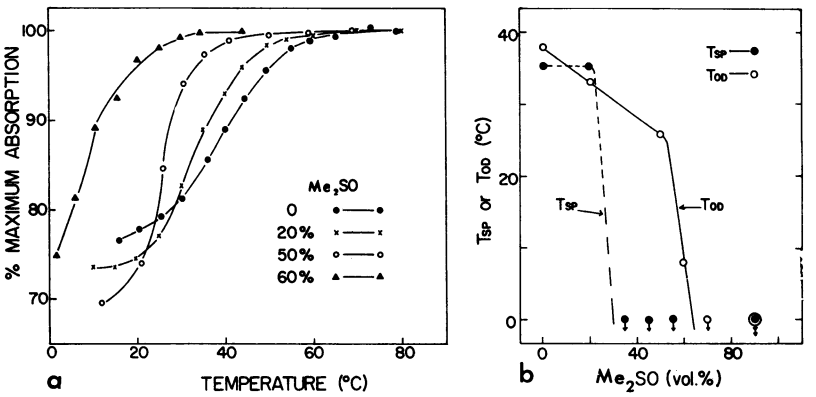


FIG. 3.—(*a*) The effect of dimethylsulfoxide (Me_2SO) on the thermal melting of tRNA as measured by the changes in absorbance at 280 mμ. (*b*) A comparison of the effects of Me_2SO on spin melting temperatures (T_{sp}) and on absorbance melting (T_{OD}). All solutions are at pH 5.

cent. Furthermore, T_{OD} does not merely decrease; the curves also sharpen with addition of Me_2SO, indicating a more cooperative behavior in the process of thermal denaturation. However, between 50 and 60 per cent there is an abrupt change in the thermal melting of tRNA in that it is markedly destabilized. Similar results have been presented for viral RNA[11] and have been observed for tRNA by circular dichroism measurements.[12]

Figure 3*b* compares the effect on the optical density and spin melting curves. It can be seen that up to 20 per cent Me_2SO has no effect on the spin melting temperature. However, at slightly greater than 20 per cent Me_2SO, T_{sp}, if it still exists, must lie below 0°, in marked contrast to T_{OD}. The effects of Me_2SO are also seen in the activation energy. At zero per cent Me_2SO, Δ_1 and Δ_2 are 5.5 and 9.8 kcal, respectively. However, at concentrations of 35 per cent and greater, the single activation energy remains constant at 7.2 kcal, significantly different from either Δ_1 or Δ_2 at lower concentrations. This sug-

gests that the denatured form of the molecule in Me_2SO is different from that of either the high- or low-temperature forms in aqueous solution.

The denaturation induced by urea is quite different from that caused by Me_2SO. As shown in Figure 4, T_{sp} and T_{OD} decrease roughly in parallel up to urea concentrations of 4 *M*. In going from 4 to 6 *M* urea, T_{OD} is further lowered from *ca.* 15°, whereas the spin melting behavior is unchanged. Figure 4*b* shows that T_{sp} decreases from 36° in the absence of urea to approximately 20° in 4 *M* urea but that further increasing the urea concentration has no additional effect, even up to a concentration of 8 *M*. The activation energies derived from the EPR melting curves also change as the urea concentration increases. There is a slight increase in Δ_1 at 4 *M* and, in going from 4 *M* to 6 *M*, Δ_1 increases and Δ_2 decreases. As a consequence, the break in the curves becomes less distinct.

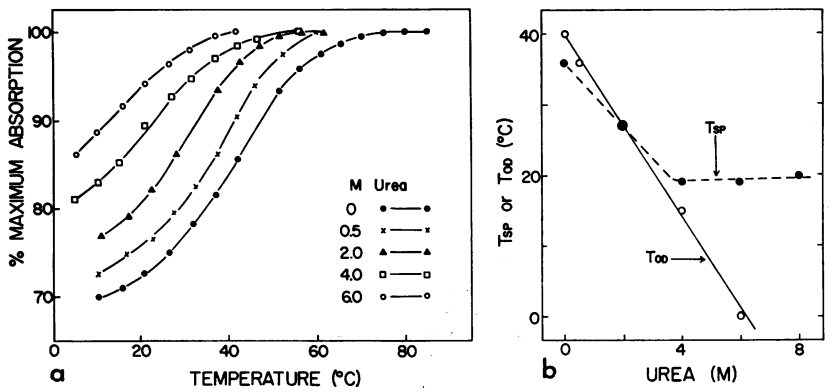


FIG. 4.—(*a*) The effects of urea on the thermal melting of tRNA as measured by changes in absorbance at 280 mμ. (*b*) A comparison of the effects of urea on spin melting and on absorbance melting. All solutions are at pH 5.

Variations in solvent viscosity do not seem to be an important factor in these measurements. The correlation time of a free nitroxide spin label is relatively insensitive to viscosity changes over the range of relative viscosities (solvent viscosity/viscosity of water at the same temperature) from 1.00 to 58[8] (the latter corresponding to 60% aqueous sucrose); the relative viscosity of 8 *M* aqueous urea is only 1.66 at 20°[13] and that of pure Me_2SO is approximately 2.

Discussion.—These experiments have demonstrated that the spin-labeled aminoacyl portion of *R*-Val-tRNA can exist in two distinct forms. There is a low-temperature form which transforms into a high-temperature form at a characteristic temperature. The sharpness of the structural transformation as measured by the spin label attached to the amino acid is in marked contrast to the gradual change in absorbance which is found when all the nucleotides in the molecule are observed. Once it is accepted that the aminoacyl end is involved in the higher-order structure of the polynucleotide chain, this sharpness is not surprising. We might expect an abrupt freeing of one end of the molecule with increasing temperature, while absorbance changes would reflect a spread

of stabilities as various regions of the molecule begin to unfold due to thermal perturbations.

The high- and low-temperature forms of *R*-Val-tRNA have different activation energies for spin mobility. In both of these forms, the motion of the spin label can reasonably be attributed to bond rotation or equivalent simple processes. The increased activation energy of the high-temperature form also deserves comment. It can be understood as resulting from the creation of a new mode of motion which can very effectively narrow the EPR lines, yet which involves, for instance, the motion of relatively bulky or hindered groups not free to move in the native, low-temperature form. The folding of the tRNA molecule is stabilized both by base stacking interactions and by hydrogen bonding. The two denaturing agents used presumably act by altering the relative contributions of these forces and, in so doing, the spin mobility. We do not have enough information to allow us to interpret these data in terms of a detailed molecular model, but the variations in spin mobility described here show that the aminoacyl end of the molecule can be freed while the bulk of the molecule is still unperturbed. This clearly suggests that the aminoacyl end, although involved in the folding, is not buried in the center of a packed globular macromolecule but rather is located near the periphery. This finding is perhaps not unexpected in view of the biological role of the adenosine terminus of the molecule in accepting amino acids and peptidyl chains.

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