A spin label study of the thermal unfolding of secondary and tertiary structure in *E. coli* transfer RNAs.

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ABSTRACT

The molecular mechanism of thermal unfolding of *E. coli* tRNA^{Glu}, tRNA^{fMet} and tRNA^{Phe} (in 0.02M Tris-HCl, pH 7.5, 10 mM MgCl₂) has been examined by the spin-labeling technique. The rate of tumbling of the spin label has been measured as a function of temperature for ten different selectively spin-labeled tRNAs. Only spin labels at position s⁴U-8 were able to probe the tertiary structure. Evidences are presented which support the hypothesis that the thermal denaturation of the three species of tRNAs studied is sequential. The unfolding process occurs in three discrete stages. The first step (30°-32°) could either be assigned to a localized reorganization of the cold-denatured structure or to a "transient" melting, followed by the simultaneous disruption of the tertiary structure and part of the hU helix. This transition is observed even in the absence of magnesium. The second step (50°-54°) involves the melting of the anticodon and miniloop regions. The last step occurs above 65° where the TψC and amino acid acceptor stems, forming one continuous double helix, melt. A simple dynamic model is considered for tRNA function in protein biosynthesis.

INTRODUCTION

In the preceeding paper (1) we have assigned the position of covalent spin-labeling on tRNA^{Glu}, tRNA^{fMet} and tRNA^{Phe} using five different spin labels. The introduction of these site specific probes provides a new approach to the problem of determining the conformation of tRNA molecules in solution. In the present paper we would like to describe our results on the temperature-induced conformational changes affecting these spin-labeled tRNAs as monitored by the change in the correlation time (τ_c) of the spin label as a function of temperature. For the three tRNAs studied, several thermal transitions were observed and suggest that all regions of the tRNA molecule do not melt out simulta-

neously, even in the presence of magnesium ions. We believe that such a selective labeling of the tRNA molecules at strategic positions enables to perceive any localized change of structure of the macromolecule upon denaturation.

Our results are compared with those obtained by other techniques giving insight into the secondary and tertiary structure of tRNA molecules in solution: NMR (2,3), fluorescence (4), temperature-jump relaxation kinetics (3) as well as thermodynamic (5) and calorimetric measurements (6).

EXPERIMENTAL

Materials and Methods

All the spin-labeled tRNAs used in this study have been described in the preceeding paper (1). The EPR spectra were obtained under conditions such that the native structure of the tRNA species is assumed to be preserved (0.02 M Tris-HCl, pH 7.5 with 10 mM MgCl₂). For the EPR spectra the concentration of the sample was about 0.5 mM in tRNA. Each value reported in Table I is the result of at least two independent temperature measurements using fresh preparation of spin-labeled tRNA.

Instrumentation

The EPR spectra were recorded on a Bruker 414S Spectrometer operating at 9.5 GHz, with a rectangular TE₁₀₂ cavity and a variable temperature control unit calibrated continuously within 1° with a Copper-Constantan thermocouple. The incident microwave power was always less than 5 mW to avoid saturation. The 100 KHz field modulation was set equal to 0.5 gauss. The samples were placed in a small (0.1 ml) aqueous flat cell (Scanlon Co.) and the thermocouple was inserted to a point just above the flat part of the cell and external to the microwave cavity. Quadratic rotational correlation times (τ_c) from EPR measurements were calculated using the simplified expression:

$$\tau_c = 6.06 \times 10^{-10} \cdot \Delta H_0 [\sqrt{h_{(0)}/h_{(-1)}} + \sqrt{h_{(0)}/h_{(+1)}} - 2] \text{ sec.}$$

where $h_{(0)}$, $h_{(+1)}$ and $h_{(-1)}$ are the amplitudes of the center, low field and high field lines respectively, and ΔH_0 is the width of the center line in gauss. The quadratic term is essentially unaffected by microwave power used in this study and the motion of the label is assumed to be isotropic (8). A linear regression

computer program was used to calculate the slopes of the Arrhenius plots. The spin enthalpy obtained is represented by the symbol H^{spin} .

RESULTS

The results are presented in the form of Arrhenius plots of the logarithm of the τ_c values against the reciprocal of the absolute temperatures and are shown in Figure 1. Refer to Fig. 7 of the previous paper (1) or to Table II of the present paper for the position of labeling on the tRNA molecules. Over the temperature range studied, from 5° to 80°, either one or two transitions are observed, as manifested by discontinuities in the slope and differences in slope value for each segment. Each segment corresponds to a particular mode of motion of the spin label; thus a particular conformation of the tRNA allows the label to move with a specific spin enthalpy. Each segment is adequately described by the mathematical expression: $\tau_i = \tau_i^0 \cdot e^{-H^{\text{spin}}/RT}$ (18). These parameters for the spin-labeled tRNAs studied are given in Table I, along with the corresponding transition temperatures. The results for MSL-tRNA (unfractionated), at the 3' end, are shown in Fig. 2.

The discontinuities in the Arrhenius plots observed on heating were completely reproducible during cooling. However, above 60° it is not possible to reproduce adequately the low-temperature spectra; the thermal denaturation changes the solvent-tRNA interactions, producing in several instances precipitation of the macromolecule. Such a phenomenon has already been observed (8).

While the absolute values (in Table I) should be regarded with caution, the relative changes accompanying each transition are of interest (see Discussion). The Arrhenius plot inflections are characteristic of a particular conformational change of the spin label-tRNA complex, and are not a general property of the free spin label. In fact, for a free spin label in water and in 75% glycerol solution, the plots are linear and monotonic over the temperature range studied with activation energies of 4.0 ± 0.2 and 9.9 ± 0.4 kcal/mole respectively. Thus, the transition temperatures observed for the spin-labeled tRNAs are not artifacts; they correspond to real physical phenomena and are consistent for the three kinds of tRNAs.

Fig. 1.

Dependence of the spin label correlation time upon absolute temperature for spin-labeled tRNA^{Glu} , $\text{tRNA}^{\text{fMet}}$ and tRNA^{Phe} in 0.02 M Tris-HCl buffer (pH 7.5), 10 mM MgCl_2 . $\bullet$ — $\bullet$, ASL; $\blacktriangle$ — $\blacktriangle$, BSL; $\blacksquare$ — $\blacksquare$, CSL; $\square$ — $\square$, HSL.

Fig. 2.

Dependence of the spin label correlation time upon absolute temperature for MSL-tRNA (unfractionated), under the same conditions as in Fig. 1

The first important feature of the present results consists in that only three principal steps for the thermal unfolding of the three species of tRNAs studied were observed by the spin-labeling technique. The first thermal transition occurs between 30° and 32° and is characteristic of tRNA molecules having a spin label at the $s^4\text{U}-8$ position, between hU and amino acid acceptor stems (ASL and BSL on $\text{tRNA}^{\text{fMet}}$ and tRNA^{Phe}). Most interesting,

TABLE I: Spin Transition Enthalpies and Transition Temperatures* for the Spin-Labeled

tRNAs					
Spin-labeled	H ₁ ^{spin}	T ₁	H ₂ ^{spin}	T ₂	H ₃ ^{spin}
tRNA	(kcal/mole)	(°C)	(kcal/mole)	(°C)	(kcal/mole)
tRNA ^{Glu}					
ASL(7)	6.1±0.6	32°	4.5±0.2	50°	5.7±0.4
BSL			8.9±0.3	53°	6.6±0.6
CSL			3.4±0.2	52°	6.3±0.6
tRNA ^{fMet}					
ASL	3.8±0.6	31°	9.3±0.5	52°	6.7±0.5
BSL	4.2±0.2	32°	7.3±0.5	52°	10.0±0.6
CSL			4.7±0.2	53°	3.1±0.5
tRNA ^{Phe}					
ASL	2.4±0.2	30°	6.1±0.4	53°	9.4±0.4
BSL	2.5±0.1(6.1)	30° (25°)	7.0±0.1(8.1)	53° (50°)	9.2±0.2 (13.3)
CSL			3.0±0.2	52°	8.1±0.9
HSL			6.7±0.2	54°	5.0±0.2
3'end MSL-tRNA (unfractionated)			5.6±0.2	65°	3.2±0.5

* These temperatures are those at which abrupt changes in the slopes of the Arrhenius plots (Fig. 1) of the motion parameters occur. These values have uncertainties of about ±2°. The values in brackets are obtained in the absence of magnesium.

is the fact that when BSL-tRNA^{Phe} is studied in the absence of magnesium (0.17M NaCl, 20mM Tris-HCl, 5m EDTA, pH 7.5) the same transition is observed.

The second transition takes place between 50° and 53° and is observed with tRNA molecules having a spin label in the anticodon region (ASL and CSL-tRNA^{Glu}, CSL-tRNA^{fMet}, CSL-tRNA^{Phe}). Only one of the tRNAs deviates from this general behavior: this is the case of BSL-tRNA^{Glu} (in the anticodon loop) where two transitions instead of one is observed. One possible explanation for this exception is that for this particular spin-labeled tRNA, the label is more sensitive to perturbations away from its site of attachment. Also, we are not excluding the possibility of an unfolding mechanism different from the general one. In either case, our results emphasize the importance of using more than one type of spin label to monitor localized structural changes in macromolecules.

Another feature of interest is the result with HSL-tRNA^{Phe} which has a label at position X-47 in the miniloop region.

Only one thermal transition is observed at 54°; it occurs either after or simultaneously with the unfolding of the anticodon region. From the X-ray crystallographic work (9,10), X-47 and s⁴U-8 residues are both situated at the apex of the extended "L"-shaped structure of the tRNA^{Phe} molecule. However, only the spin-labeled s⁴U-8 residue sees the first transition at 30°-32°. Consequently, an interesting conclusion to be drawn from this difference in behavior is that the spin-labeled s⁴U-8 residue seems to be able to monitor changes in tertiary structure of the tRNA molecule, since this residue is directly involved in a tertiary hydrogen-bonding with residue A-14 of the hU stem. The situation is different with spin-labeled residue X-47 which is located in a non base-paired region (9,10) and more exposed to the solvent than the s⁴U-8 residue (1). The active tertiary structure of the tRNA molecule is very likely still preserved when the spin label is covalently linked to the s⁴U-8 residue, since similar modified tRNAs have shown to retain their original amino acid acceptance activities (11,4).

Another interesting observation is that tRNAs having a spin label at the s⁴U-8 position always see both transitions. This suggests that the region around s⁴U-8 is quite rigid and the label is able to monitor the subsequent dissolution of the remaining ordered regions of the partially melted macromolecule. In contrast, labels in the anticodon and miniloop regions see only one transition at ~52°, perhaps because these regions are more flexible and therefore not able to perceive other transitions (see the model in Discussion).

The third and last step in the unfolding process was observed with MSL-tRNA (unfractionated), labeled at the 3' end (Fig. 2). The transition is at 65° and parallels the data obtained with Val-tRNA^{Val}, where the spin label was bound to the α-amino group of the attached amino acid (12). In the latter case, the transition takes place at a temperature 5° higher than ours, but such a difference is certainly due to differences in the structure and location of the spin label. Since the acceptor stem of many tRNAs contains a large proportion of G-C base pairs, it is realistic to expect that this helix would be the last one to melt and consequently would have the highest spin transition temperature (Table I). Introduction of an iodoacetamide spin label into a C-75

modified residue of tRNA^{Phe} from yeast has been reported and a single melting transition at 47° was observed (8). Sprinzl et al (8) identified this transition to a cooperative process, not associated with a local perturbation of the 3' end, but related to a global conformational change of the extended "L"-shaped tRNA molecule. From the preceding arguments we realize that their conclusion can hardly be accepted.

Unfortunately, we were not able to spin label the T Ψ C region; X-ray work (9,10), however, has shown that the T Ψ C loop is actively involved in tertiary bonding, whereas the anticodon loop is not. Other studies indicate that the T Ψ C stem is quite stable and is not exposed to the solvent (13). The amino acid acceptor and T Ψ C helices being colinear and juxtaposed to form one continuous helix, very likely melt simultaneously (6).

DISCUSSION

Using temperature-jump relaxation kinetics, up to five cooperative melting transitions for E.coli tRNA^{fMet} (without magnesium) were observed and the authors attempted to correlate these with spectral changes in NMR experiments (3). With yeast tRNA^{Phe} (without magnesium), five transitions have also been resolved by differential UV absorption (14). However, in the presence of magnesium, which is imperative for an understanding of the biologically active structure of tRNA, we do not see as many transitions using spin-labeled tRNAs; unless of course if these transitions were unreflected in measurable changes in the movement of the spin label.

The molecular mechanism, proposed by Crothers et al (3), for the thermal unfolding of tRNA^{fMet}, in the absence of magnesium, is such that at 30° part of the hU helix can open transiently, followed by the simultaneous disruption of "tertiary interactions" and the rest of the hU helix (46°), and then, in succession, T Ψ C (61°), anticodon helix (70°) and finally the acceptor stem (77°). On the other hand, the NMR work of Kearns and coworkers (2) argues that, without magnesium, the hU stem and tertiary structure melt more or less simultaneously at ~45° whereas the anticodon, T Ψ C and amino acid acceptor stems melt between 56° and 70°. In the presence of magnesium, however, they observed a perturbation of the spectra at 25°, followed at 60° by the unfolding of hU and anti-

codon regions and finally full denaturation occurs between 68° and 85°.

It is possible that the value obtained for our first transition in the presence (~30°) or in the absence of magnesium (25°) corresponds to the "transient" transition observed by temperature-jump relaxation kinetics (3). However, it is at variance with the possibility of a magnesium-induced conformational change (2). In fact, the thermodynamic studies of Levy and Biltonen (15) strongly suggest that magnesium ions do not directly induce any thermodynamically significant conformational change upon binding to tRNA but insure that the folded biologically active form of tRNA is more stable than any other forms. On the EPR spectra, addition of magnesium has the effect of increasing the τ_c values of the spin labels, corresponding to a more compact structure of the tRNA (unpublished results). Furthermore, our results have the additional advantage of localizing this low temperature transition near the s⁴U-8 residue. Therefore, this transition could possibly be assigned to a "transient" melting that could be immediately followed by the simultaneous disruption of the tertiary structure and part of the hU helix.

Another alternative that cannot be neglected is that tRNA molecules were shown also to be reversibly unfolded upon cooling (15). The perturbation seen at ~30° might then reflect a structural reorganization for the refolding of the tRNA molecule to its native form. This structural reorganization could involve some tertiary hydrogen bonds as well as part of the hU helix but without loss of the active tertiary structure. And finally, a particular structure of water molecules around tRNA below 30° could also be in part a consequence of this low melting transition. Further work is oriented into this direction.

Consider now early melting transition in tRNA molecules in terms of the loss of the tertiary structure, and also melting of the helix. In the cloverleaf structure, the s⁴U-8 residue is placed in a non-paired region between the amino acid acceptor and hU regions. The s⁴U-8 residue is also involved in a tertiary hydrogen-bonding with residue A-14 (9,10). Thus, the EPR spectra of spin-labeled tRNAs having a spin label at this position are expected to be strongly dependent on its environment (20) and it is natural to expect that the structure disrupted in the first

thermal transition involves first non-cloverleaf binding, or tertiary structure. This local perturbation could be immediately followed by a loss in the secondary structure of the nearby hU helix. Such a conclusion follows already from the optical experiments of Cole and Crothers (16). Although this suggestion is at variance with results obtained by differential UV techniques (14), it agrees with the interpretation first given by Fresco *et al* (17), largely on the basis of hydrodynamic measurements.

The second transition begins at around 50° and is very likely associated to the final unfolding of the rest of hU helix, followed by the modification of the secondary structure of the anticodon helix, presumably involving initially the breakage of few base pairs, and then the unwinding of the miniloop region (at 54°). This transition is then followed by the last step in the thermal denaturation process; the melting of T ψ C and amino acid acceptor stems above 65°.

The spin labeling technique has the beneficial possibility of being able to monitor localized structural perturbations because of the presence of a marker at a specific region. Consequently, the present EPR data favor a mechanism whereby, for the three tRNAs studied, the anticodon region melts before the T ψ C and acceptor stems. Although, optical, NMR and EPR melting phenomena may not have to correspond exactly, our transitions are in the same range of temperature (2,16,6). This further indicates that the introduction of a spin label does not seem to change appreciably the native conformation of tRNA molecules with respect to both secondary and tertiary structure. Therefore, from the present EPR data, it is clear that the thermal unfolding of tRNA, in the range of temperature from 5° to 80°, passes through three discrete stages in the presence or absence of magnesium. This process may be a common feature for all tRNA molecules. Because of the large spread of temperature (15° to 20°) between each transitions, the melting process appears to be sequential rather than cooperative, although a certain degree of cooperativity could be involved within the sharp transitions (a spread of only 3°).

A very important problem in the present study concerns the different energy values for the spin mobility. Let us say first that the slope of a graph of $\log \tau_c$ vs $1/T$ (as in Figs. 1

and 2) does not yield an activation energy related to a structural change. It yields an energy related to the hindrance of the spin label which we have named the spin enthalpy for distinction. Increasing temperature mobilizes the spin label against this steric factor. Conformational changes are detected when there is an inflection in the graph; the inflection indicates a change in the barrier. This is what we have been observing with our spin-labeled tRNAs. However, if a continuous (i.e. cooperative) conformational change were induced by increasing temperature, $\log \tau_c$ vs $1/T$ would not be a straight line nor a sequence of straight lines. The experimental results shown in Figs. 1 and 2 prove unequivocally a sequential rather than a cooperative process for the thermal unfolding of tRNA molecules (6).

Inspecting further Fig. 1, we find that in general, for the same interval of temperature, the change in τ_c is larger at high than at low temperatures. Thus the barrier to rotation of the label is larger at higher temperatures, implying more steric constraint on the label by its surrounding environment. By unfolding the tRNA molecule, base stacking interactions and hydrogen bonds are broken, the spin mobility increases in consequence, and this in spite of the fact that the denatured state of tRNA has a more extended form (less compact and more bulky) than the native structure (18,19). The variations in the slope at high temperatures could mean that some tRNAs are more prone to aggregation.

What now appears as more relevant to a better understanding of the structure of tRNA molecules and the unfolding process are the differences (ΔH^{spin}) in the spin transition enthalpies between two melting transitions. These values, summarized in Table II, are directly related to the rate of the tumbling motion that the spin label "feels" in going from one environment to another and are independent of any external factor. We argue that the smaller the value, the smaller is the change in conformational fluctuation in going from one state to another, in other words, the greater is the flexibility of the molecule in this region. It is noteworthy that the largest differences (3.1 to 5.5) are observed when the spin label is on the s^4U-8 residue, whereas the smallest values (1.2 to 2.9) are obtained for the spin label at the CCA end, the anticodon and miniloop regions. Only CSL-tRNA^{Phe} does

not fit into this general pattern. Despite this irregularity, analysis of the data leads us to propose a plausible dynamic model, that could have possible importance for tRNA function in protein biosynthesis. While our model is based solely on EPR evidences it leads to usefull predictions that can be tested further. From comparison of the ΔH^{spin} values (Table II) we propose that, in solution, the CCA-end, the anticodon and miniloop regions of tRNA are more flexible (small ΔH^{spin}) than in the region around the $s^4\text{U}-8$ residue (larger ΔH^{spin}). On the other hand, the continuous double helix composed of amino acid acceptor and T ψ C stems, which melts last, should be much more rigid than the rest of the molecule. An appealing feature of this semi-flexible structure is that it fits in well with the proposals of Woese (21) and Levitt (22); according to them several structural changes in the anticodon loop should facilitate its contact with the m-RNA in the ribosome. The flexibility of the CCA end to allow transfer of the growing polypeptide chain meets again the proposition of Gurel (23). The second proposition in our model, concerning the rigid portion (T ψ C and acceptor stems), is also important, this form the point of view of good fixation and recognition purpose on the ribosomal sites (24, 25).

TABLE II: Differences in Spin Transition Enthalpies Between Melting Transitions for the Spin-Labeled tRNAs (obtained from Table I).

Spin-labeled tRNA	$\Delta H^{\text{spin}}_{2,1}$ (kcal/mole)	$\Delta H^{\text{spin}}_{3,2}$ (kcal/mole)	Location of the Label
<u>tRNA^{Glu}</u>			
ASL	+2.8	+1.2	(s ² U*-35) anticodon
BSL		-2.3	(s ² U*-35) anticodon
CSL		+2.9	(s ² U*-35) anticodon
<u>tRNA^{fMet}</u>			
ASL	+5.5	-2.6	s ⁴ U-8
BSL	+3.1	+2.7	s ⁴ U-8
CSL		-1.6	(U-37) anticodon
<u>tRNA^{Phe}</u>			
ASL	+3.7	+3.3	s ⁴ U-8
BSL	+4.5	+2.2	s ⁴ U-8
CSL		+5.1	(U-33) anticodon
HSL		-1.7	(X-47) miniloop
3'end MSL-tRNA (unfractionated)		-2.4	3'-CCA end

In conclusion, we have demonstrated that the spin-labeling technique is a useful method to probe the melting behavior of species such as tRNAs. Further application of this technique should prove to be exceedingly useful to study conformational characteristics of local regions of tRNA during the various steps of protein biosynthesis, such as described by our dynamic model. More experiments along these lines are now in progress.

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REFERENCES

- 1) Caron, M. and Dugas H. (1975) Nucleic Acid Res., **3**, 19-34.
- 2) Wong, K.L. Wong, Y.P. and Kearns, D.R. (1975) Biopolymers, **14**, 749-762.
- 3) Crothers, D.M., Cole P.E., Hilbers, C.W. and Shulman, R.G. (1974) J. Mol. Biol., **87**, 63-88.
- 4) Yang, C.H. and Söll, D. (1974) Proc. Nat. Acad. Sci. U.S., **71**, 2838-2842.
- 5) Coutts, S.M., Gangloff, T. and Dirheimer, G. (1974) Biochemistry, **13**, 3938-3948.
- 6) Brandts, J.F., Jackson, W.M. and Yao-Chung Ting, T. (1974) Biochemistry, **13**, 3595-3600.
- 7) McIntosh, A.R., Caron, M. and Dugas, H. (1973) Biochem. Biophys. Res. Commun., **55**, 1356-1363.
- 8) Sprinzl, M., Krämer, E. and Stehlik, D. (1974) Eur. J. Biochem., **49**, 595-605.
- 9) Kim, S.H., Sussman, J.L., Suddath, F.L. Quigley, G.T., McPherson, A., Wang, A.H.J., Seeman, N.C. and Rich, A. (1974) Proc. Nat. Acad. Sci. U.S., **71**, 4970-4974.
- 10) Robertus, J.D., Ladner, J.E. Finch, J.T., Rhodes, D., Brown, R.S., Clark, B.F.C. and Klug, A. (1974) Nature, **250**, 546-551.
- 11) Hara, H., Horiuchi, R., Sanayoshi, M. and Nishimura, S. (1970) Biochem. Biophys. Res. Commun., **38**, 305-311.
- 12) Schofield, P., Hoffman, B.H. and Rich, A. (1970) Biochemistry, **9**, 2525-2533.
- 13) Gralla, J. and Crothers, D.M. (1973) J. Mol. Biol. **72**, 497-511.
- 14) Römer, R., Riesner, D., Coutts, S.M. and Maass, G. (1970) Eur. J. Biochem., **15**, 77-84.
- 15) Levy, J. and Biltonen, R. (1972) Biochemistry, **11**, 4145-4152.
- 16) Cole, P.E. and Crothers, D. (1972) Biochemistry, **11**, 4368-4374.
- 17) Fresco, J.R., Adams, A., Ascione, R., Henley, D. and Lindahl, T. (1966) Cold Spring Harb. Symp. Quant. Biol. **33**, 527-538.

- 18) Hoffman, B.M., Schofield, P. and Rich, A. (1969) Proc. Nat. Acad. Sci. U.S., 62, 1195-1202.
- 19) Goldstein, R.N., Stefanovic, S. and Kallenbach, N.R. (1972) J. Mol. Biol., 69, 217-236.
- 20) Dourlent, M., Yaniv, M. and Hélène, C. (1971) Eur. J. Biochem., 19, 108-114.
- 21) Woese, C. (1970) Nature, 226, 817-820.
- 22) Levitt, M. (1973) J. Mol. Biol. 80, 255-263.
- 23) Gurel, D. (1973) Physiol. Chem. & Physics, 5, 177-182.
- 24) Pongs, D., Nierhaus, K.H., Erdmann, V.N. and Wittmann, H.G. (1974) FEBS Letters, 40, S28-S37.
- 25) Dube, S.K. (1973) FEBS Letters, 36, 39-42.