A modulation-free Electron Spin Resonance spectrometer was used for the registration of spectral absorption lines of a spin-labeled Escherichia coli phenylalanine tRNA in solution with low (less than 0.1%) line shape distortion. The analysis of line shape of two different spin-labels introduced into position 8 revealed that phenylalanine tRNA in solution exists as a mixture of two conformers, the equilibria between conformers being dependent on pH, concentration of magnesium and functional state of tRNA (deacylated, aminoacylated or peptidylated). There are no overall structural rearrangements upon aminoacylation or peptidylation of tRNA. The observed small changes of spectral line shape can be assigned to shifts in conformational equilibria.

INTRODUCTION

In models of protein synthesis (1,2) and theories of the development of protein synthesis during evolution (3,4) conformational changes of tRNA molecules play a crucial role. But experimental data on functionally relevant conformational changes of tRNA's are ambiguous and conflicting even in the simplest case of tRNA aminoacylation (see review (5)). Introduction a nitroxyl spin labels into specific sites of tRNA (6) enables to apply ESR spectroscopy in the study of tRNA conformation in solution (7,8) and in the state of adsorption on ribosomes (9). High precision of registration and line shape analysis are necessary for correct studies of conformational transitions by the ESR method. A precise registration of line shape in widely used ESR spectrometers with modulation of the magnetic field is possible only if the modulation amplitude is less than the half width of absorption lines. This results in a significant decrease of sensitivity.

Using a new method of ESR detection without modulation (10),
a new spectrometer was built for measurement of the absorption line with both high sensitivity and precision (11). The question of whether aminoacylation induced a conformational change in tRNA was reinvestigated.

We found no gross changes in tertiary structure of tRNA both upon aminoacylation and in Mg\textsuperscript{2+} induced isomerization of tRNA, resulting in drastic changes of tRNA affinity to ribosomes (12, 13). In the same time the ESR spectra revealed that in all conditions tRNA exists as a mixture of conformers. The balance of tRNA conformational states is mediated by aminoacylation or peptidylation and ionic conditions.

MATERIALS AND METHODS

Escherichia coli tRNA was prepared according to Zubay (14), aminoacylated by \([^{14}C]\)phenylalanine with a specific activity 318 mCi/mmol (UVVR, CSSR) and was enriched till 1500 pmoles of phenylalanine per A\textsubscript{260} unit according to Gillam and Tener (15).

Two deuterated spin-labels were used (fig. 1) for labeling tRNA\textsubscript{Phe}: \(3-(2\text{-bromoacetamido})-2,2,5,5\text{-tetramethylpyrrolidin-1-oxyl}\) (label I) and acetobromid-\(2,2,6,6\text{-tetramethyl-4-aminopiperidin-1-oxyl}\) (label II). The synthesis of spin labels was performed according to Rozantzev (16) with some modifications: 2,2,6,6-tetramethylaminopiperidin was converted into a base and then oxidized. Acetyl groups were removed by boiling in 15\% alkali up to full dissolving of the pellet and oil. Label II was bromacetylated according to (17). The use of spin-labels with deuterium instead of hydrogen results in the decrease of the contribution

Fig. 1. Chemical structures of spin-labels used.
of nonresolved hyperfine protons splitting to the line shape.

Spin-labeling of tRNA\textsuperscript{Phe} was performed according to Caron and Dugas (6) except the addition to the reaction mixture of 5 mM EDTA. The reaction was followed by measurements of absorption at 330 nm characteristic for s\textsuperscript{4}U. The reaction mixture was neutralized to pH 7.0 by addition of 1 M sodium acetate buffer pH 5.0. The spin-labeled tRNA was precipitated by addition of cold ethanol. The dissolving and precipitation by ethanol was repeated several times until a coincidence of tRNA concentration (A\textsubscript{260}) and spin-label concentration (ESR measurements). In final preparations there were no signs of spectra of free spin-labels. A ruby-monocrystal particle placed into the cell of ESR spectrometer was used as absolute spin concentration standard. Calibration was made using aqueous solution of spin-label II and Cu\textsuperscript{2+}.

The enzymatic hydrolysis of spin-labeled tRNA\textsuperscript{Phe} by RNase T\textsubscript{1} (Freehold, New Jersey) and analysis by chromatography according to (6) revealed that all spin-labels were only in the oligonucleotide containing s\textsuperscript{4}U.

The spin-labeled tRNA\textsuperscript{Phe} was aminoacylated by [\textsuperscript{14}C]phenylalanine up to 700-900 pmoles of phenylalanine per 1 unit A\textsubscript{260} and was separated from nonacylated tRNA\textsuperscript{Phe} by chromatography on DE-cellulose (16). Nonacylated and aminoacylated spin-labeled tRNA\textsuperscript{Phe} was eluted in 12 and 17 per cent of ethanol respectively. According to [\textsuperscript{14}C]radioactivity and ESR absorption measurements 85% to 95% of Phe-tRNA\textsuperscript{Phe} were spin-labeled.

Spin-labeled peptidyl-tRNA (N-acetyl-Phe-tRNA\textsuperscript{Phe}) was prepared by acetylation of spin-labeled [\textsuperscript{14}C]Phe-tRNA\textsuperscript{Phe} according to Rappoport and Lapidot (18). The extent of acetylation was higher than 95%. All preparations were stored in liquid nitrogen.

Before ESR measurements tRNA was dialyzed against the desirable buffer with EDTA or magnesium and heated at 35° for 5 min. The buffers used were: 50 mM sodium acetate in the range of pH 4.0-5.0, 100 mM bis-tris in the range of pH 6.0-7.0 and 100 mM tris-HCl pH 7.0-8.0.

The design and construction of the ESR spectrometer using a method of registration without modulation of the magnetic field (10) was described earlier in (11). In this instrument the mag-
Nucleic Acids Research

Nucleic field is periodically scanned in the observed spectrum range. The output signal in this case is a repeating undistorted ESR spectrum with an amplitude equal to the amplitude of the absorption line. The signals are amplified by broad-band amplifier with the passband chosen to provide a particular value of the signal distortion. Averaging of a signal is performed simultaneously with the magnetic field scanning. This method was realized by using of a bimodal cavity described in (13), exploiting the Faraday effect. The working frequency of the spectrometer is 8,700 MHz. The spectrometer can register both the absorption and dispersion signals with the same high sensitivity. The volume of the cell is 0.1 ml. The scanning period is 50 msec. For nitroxyl radicals in aqueous solution at $10^{-4}$ M concentration the S/N ratio is 50 for one 50 msec scan. The accuracy of the line record is not less than $10^{-3}$ in rms terms. The working concentrations of spin-labeled tRNA were in the range of $(2-5)\times10^{-5}$ M, the number of scans for registration was 1000.

A typical ESR absorption spectrum of spin-labeled tRNA recorded by the modulation free ESR spectrometer is shown on fig.2.

We see that it is well resolved triplet characteristic of rapidly tumbling spin labels ($\tau \sim 10^{-9}$ sec). Consequently the calculation and analysis of line shape was performed according to the theory of rapid motion (20).

Fig.2. ESR spectrum of a spin-labeled tRNA with the definition of the parameters for line shape characterization.
The output digital information from the modulationfree spectrometer ([11]) was treated by a computer M 4030 (analog of IBM 300/4). Due to good resolution of the spectra, we determined the centers of the lines, suggesting their symmetry, and then every line was approximated by an even function:

\[ y = \frac{F_{\text{max, exper}} \cdot \alpha_2}{1 + \alpha_2 H^2 + \alpha_3 H^4 + \alpha_4 H^6} \]  

where \( F_{\text{max, exper}} \) is the maximum value of the experimental line, \( \alpha_1, \alpha_2, \alpha_3, \alpha_4 \), are approximate coefficients. Approximation was performed from \( 1/3 F_{\text{max}} \) to \( F_{\text{max}} \) (see fig.2) using a computer programm FUMILI for the minimization of weighted square deviations.

The resulting function was used for the calculation of half widths: at the levels \( 1/3 F_{\text{max}} - \Delta H_{1/3} \), \( 1/2 F_{\text{max}} - \Delta H_{1/2} \), \( 2/3 F_{\text{max}} - \Delta H_{2/3} \) and at the point of maximum derivatives - \( \Delta H_{p-p} \) as shown on fig.2.

Both rotational correlation times \( \tau_c^{(1)} \) and \( \tau_c^{(2)} \) determining the degree of motion anisotropy of spin-labels were calculated as in (22). The spin Hamiltonian parameters were taken from (23). Besides the determination of correlation times the line shape in each spectrum was analyzed by comparing half widths at different heights. For rapid motion the line shape must be a Lorentz function, for which

\[ \Delta H_{1/3}/\Delta H_{2/3} = 2; \quad \Delta H_{1/2}/\Delta H_{p-p} = 2.45 \]  

All half widths used for calculation of \( \tau_c^{(1)} \) and \( \tau_c^{(2)} \) and for analysis of line shapes were obtained by extrapolation of power dependence of half widths to zero power.

RESULTS AND DISCUSSION

Fig. 3 shows the pH dependence of correlation times \( \tau_c^{(1)} \) (a) and \( \tau_c^{(2)} \) (b) for spin-labeled decylated tRNA\textsuperscript{Phe} (label II) in the presence of EDTA or magnesium. A similar dependance was observed for spin-label I. We can see from fig. 3 that observed correlation times are in the range \( 10^{-9} \) sec, i.e. in the range of rapid motion. So the shape of every line in the triplet must be a Lorentz function (20). To verify this we simulated theoretical spectra in the observed range of correlation times
using a computer program from (23) and made the same analysis of line shapes as for experimental spectra. No deviation from Lorentz function in line shapes of computer simulated ESR spectra was observed.

The results of analysis of experimental spectra for tRNA\textsuperscript{Phe} (label II) are shown on fig. 4. Qualitatively the same results were obtained with label I (not shown). The main feature of all experimental spectra of spin-labeled tRNA\textsuperscript{Phe} is the deviation of line shape from Lorentz function. One can see on fig. 4 that experimental lines decline slower than theoretical ones. Thus the ratios of halfwidths $\Delta H_{1/2}/\Delta H_{2/3}$ and $\Delta H_{2/3}/\Delta H_{p-p}$ exceed the Lorentzian values 2.0 and 2.45 respectively.

On the opposite measurements in a model system (label I in viscous solution of sucrose) in the range of correlation times about $10^{-9}$ sec also did not reveal any deviations from Lorentzian shape.

Comparing the analysis of experimental spectra, with computer simulated ones we suggest that the anomalies in line shapes of spin-labeled tRNA spectra are due to heterogeneity in con-
Fig. 4. The dependence of line shapes on pH for tRNA^{Phe} (label I)
a - for $\Delta H_{1/2} / \Delta H_{2/3}$, b - for $\Delta H_{1/2} / \Delta H_{P-P}$,
1 - in the presence of 1 mM EDTA, 2 - in the presence of 10 mM magnesium.

formation states of tRNA in solution. We can see on fig. 4 that the highest deviations from Lorentzian shape in buffers without magnesium are at low pH for components $m_I = +1$ and $m_I = -1$ and lower at neutral pH. The presence of magnesium ions increases the deviations from Lorentzian shape at any pH, the dependence on pH being less pronounced. The difference between spectra in the presence and in the absence of magnesium increases at low temperatures.

Fig. 5a demonstrates ESR spectra ($m_I = 1$) of deacylated tRNA^{Phe} (label II) in the absence (1) and in the presence (2) of
magnesium at 6°C. We can see without computations the appearance of an asymmetric shoulder in the low field component in the presence of magnesium ions. Such deviations usually imply the superposition of conformational states. Indeed the experiments shown on fig. 5b and c confirm this suggestion. ESR spectra of tRNA^Phe at different temperatures revealed many isobestic points in the presence of magnesium (fig. 5 b, c). As was shown in (24) the presence of more than one isobestic point in spectra leads to the assumption that the system contains two component. Thus deacylated spin-labeled tRNA in solution at observed temperatures consist presumably of two conformers. The equilibrium between conformers depends on environmental condition (pH, Mg^{2+}, temperature).

Taking into account these data we compared the spectra of
deacylated tRNA^{Phe}, Phe-tRNA^{Phe} and N-acetyl-Phe-tRNA^{Phe} (label I). Fig. 6 shows that in the absence of magnesium (curves 1 a, b, c) the line shapes are close to Lorentz function with a correlation time $\tau_c^{(2)} = 3.5 \times 10^{-9}$ sec independent of biological state of tRNA.

In the presence of magnesium for all tRNA's (fig. 6 a, b, c, curves 2) we can see the appearance of large asymmetry for the low field component and slower decline for the high field component. This again implies the presence of different conformations of spin-labeled tRNA's. The existence of isobestic points in ESR spectra for N-acetyl-Phe-tRNA^{Phe} (fig. 7) confirms the existence of a mixture of two conformational states of N-acetyl-Phe-tRNA^{Phe} in solution.

It was impossible to use the correlation time values to dis-

---

**Fig. 6.** Comparison of spectra for tRNA^{Phe}, Phe-tRNA^{Phe} and N-acetyl-Phe-tRNA (label I) at 6°C in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl$_2$ or 1 mM EDTA.

- a = tRNA^{Phe}; b = Phe-tRNA^{Phe}; c = N-acetyl-Phe-tRNA^{Phe}
- 1 = 1 mM EDTA, 2 = 10 mM MgCl$_2$; 3 = 10 mM MgCl$_2$.
Fig. 7. ESR spectra of N-acetyl-Phe-tRNA$_{\text{Phe}}$ at different temperatures (a). b - components $m_I = +1$, c - components $m_I = -1$, in other scale.

tinguish conformational changes during aminocacylation. Only comparative analysis of line shapes revealed a difference between deacylated and aminocacylated tRNA and a less pronounced difference between tRNA and peptidyl-tRNA (fig. 6d).

On the basis of these data we can affirm that structural changes upon aminocacylation (at least near the thiouracyl group of tRNA) are rather small.

On the other hand, if the mixture of conformational states of tRNA is real, than the changes in spectra of tRNA, aminocacyl- or peptityl-tRNA are the result of conformational equilibrium shifts or variations in rates of conformers interconversion.

Fig. 8 shows the ESR spectra of tRNA$_{\text{Phe}}$, Phe-tRNA$_{\text{Phe}}$ and N-acetyl-Phe-tRNA$_{\text{Phe}}$ for the same preparations as in fig. 6d but normalized to spin concentration. The observed isobestic points in this case strongly suggest the interconversion of conformational states during tRNA$_{\text{Phe}}$ - Phe-tRNA$_{\text{Phe}}$ - N-acetyl-Phe-tRNA$_{\text{Phe}}$.
Fig. 8. Comparison of ESR spectra of different forms of tRNA.
- a condition as for fig. 6d but normalized to the spin concentration;
- b component $m_I = +1$; 1 - tRNA$^{\text{Phe}}$
- 2 - Phe-tRNA$^{\text{Phe}}$
- 3 - N-acetyl-Phe-tRNA$^{\text{Phe}}$

transitions. Unfortunately, we can not yet present a quantitative estimation of their interconversion.

We considered as an alternative to our interpretation of experimental data a highly anisotropic motion of the spin-label in case when the molecular axes and the principal axes of the diffusion tensor do not coincide. The simulation of corresponding ESR spectra using the program from (23) did not show the observed deviation in spectral line shapes. We are aware nonetheless that the program used has limitation in the synthesis of ESR spectra.

It is worth noting that we did not find any changes in overall structure of tRNA (at least in the central part of tRNA molecule) in the presence or the absence of magnesium (12,13).

Nevertheless, the tRNA molecules according to our data exist in solution as a mixture of conformers (most probably in two states). The environmental conditions and biological state of
tRNA (aminoacylation, peptidylation) shift the equilibrium between these conformers.

There is accumulating evidence in literature that tRNA exists in solution in many interconvertible conformations (review 5). Three laboratories have concluded after examination of NMR spectra of the methyl group in magnesium containing solutions that there must exist more than one native conformer of yeast tRNA$_{Val}$ and tRNA$_{Phe}$ (26,27). Reid (26) suggested from NMR data the existence of two tRNA conformational states.

Multiple tRNA conformational states are also indicated by some works on native yeast tRNA$_{Phe}$, with ethidium bromide covalently attached either to the dihydouridine residues in the D-loop or to the Y-base with preservation of biological activity (28,29,30). In tRNA$_{Phe}^{E}t_{6}^{16/17}$ the ethidium is present in three states which are distinguished by their fluorescence lifetime (28,29). The distribution of these states was found to depend on the concentration of Mg$^{2+}$ and can be changed by the cognate synthetase (30). Similar conclusions were made in studies of conformational changes of the anticodon region of tRNA$_{Phe}$ (31,32).

Our data are in accord with all these observations. The spin labeled S$^4$U-8 residue is the most sensitive probe to monitor changes in tertiary structure of the tRNA molecule, since this residue is directly involved in tertiary hydrogen bonding with residue A-14 of the hU stem. The modulation free spectrometer enables us to measure very subtle changes in ESR spectra of tRNA. We do not know the exact nature of difference between observed tRNA conformers. It is possible that these changes are similar with low temperature "transient" melting near the S$^4$U-8 residue observed in (7).

Acknowledgement. We are indebted to Mrs Popova M.G. and Mrs Krzyszkaja G.I. for their invaluable help on ESR spectral measurements and for the chemical synthesis and Dr. Makhno V.I. for the isolation of total tRNA from E.coli.
REFERENCES

1. Woese, C.K. (1973) Nature, 226, 817-820.
2. Lake, J.A. (1977) Proc. Natl. Acad. Sci. USA, 74, 1903-1907.
3. Crick, F.H.C., Brenner, S., Klug, A., Picczenik, G. (1976), Origins of Life, 7, 389-397.
4. Eigen, M., Schuster, P. (1978), Naturwissenschaften, 65, 341-369.
5. Crothers, D.M., Cole, P.F. (1978) in Transfer RNA, ed. by S. Altman, pp 196-247, MIT Press, Cambridge, Massachusetts.
6. Caron, M., Dugas, H. (1976) Nuc. Acids Res., 3, 1903-1907.
7. Caron, M., Dugas, H. (1976) Nuc. Acids Res., 3, 35-47.
8. Caron, M., Brisson, N., Dugas, H. (1976) J. Biol. Chem., 251, 1529-1536.
9. Rodriguez, A., Tougas, J., Brisson, N., Dugas, H. (1980) J. Biol. Chem., 255, 8116-8120.
10. Isaev-Ivanov, V.V., Lavrov, V.V., Fomichev, V.N. (1976) Dokl. Acad. Nauk SSSR, 229, 70-72.
11. Anisimov, G.K., Zavazsky, E.I., Isaev-Ivanov, V.V., Lavrov, V.V., Fomichev, V.N. (1981) J. Tech. Phys. (Leningrad), 21, N 5, 985-987.
12. Kirillov, S.V., Makhno, V.I., Odinzyov, V.B., Semenkov, Yu. (1978) Eur. J. Biochem., 89, 305-313.
13. Kirillov, S.V., Odinzo, V.B. (1978) Nucl. Acids Res., 5, 1501-1514.
14. Zubay, G. (1962) J. Mol. Biol., 4, 347-356.
15. Gillan, I.C., Tener, J.M. (1974) in Methods in Enzymology, Vol. XX part C, pp 55-70, Academic Press, New York.
16. Rozantsev, E.G. (1970) "Free iminopyr radicals", Khimia, Moskwa, 199-200.
17. Price, N.G. (1973) FEBS Letters, 36, 351-354.
18. Rappoport, S., Lapidot, Y. (1974) in Methods in Enzymology, Vol. XXIV part E, pp 685-963, Academic Press, New York.
19. Isaev-Ivanov, V.V., Fomichev, V.N. (1976) Pribory i tehnika experimenta (Moskwa), N 3, 172-173.
20. Freed, T.N., Fraenkel, G.R. (1963) J. Chem. Phys., 39, 326-348.
21. "Statistical methods for the experimental physics" ed. by A.A. Topkin (1976) pp 319-325, Atomizdat, Moskwa.
22. Dwek, R.A. (1973) "Nuclear magnetic resonance in biochemistry", pp 285-327, Clarendon Press, Oxford.
23. "Spin labeling: Theory and applications" ed. by L.J. Berliner (1976) pp 64-155, Acad. Press, New York.
24. Bernstein, I.J., Kaminski, Yu.L. (1975) "Spectrophotometric analysis in organic chemistry" pp 35-42, Khimia, Leningrad.
25. Kan, L.S., Tso, P.O.P., von der Haar, F., Sprinzl, M., Cramer, F. (1975) Biochemistry, 14, 3278-3291.
26. Reid, B.R. (1977) in Nucleic acid-Protein recognition ed. by H.F. Vogel, pp 375-390, Acad. Press, New York.
27. Kastrup, R., Schmidt, P.G. (1975) Biochemistry, 14, 3612-3618.
28. Rigler, R., Ehrenberg, M., Wintermeyer, W. (1977) in Molecular Biology, Biochemistry and Biophysics, vol. 24, pp 219-244, Springer Verlag, Heidelberg.
29. Ehrenberg, M., Rigler, R., Wintermeyer, W. (1979) Biochemistry, 18, 4588-4599.
30. Rigler, R., Nilson, L., Wintermeyer, W., Pachman, V., Zachau, H.G. (1981) Nucl. Acids Res., 9, 1031-1044.
31. Urbance, C., Maass, G. (1978) Nucleic Acids Res., 5, 1551-1560
32. Ehrlich, R., Lefevre, J.F., Remy, P. (1980) Eur. J. Biochem., 103, 145-153.