4THIOURIDINE-SPECIFIC SPIN-LABELING OF *E. coli* TRANSFER RNA

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Studies on conformational structure have become very important to understand the biological functions of tRNA, because it is proving difficult to explain the specificities of tRNA on the basis of simple differences in nucleotide sequence (1-3). Recently spin-labeling has been explored as a technique to study the conformational and functional properties of nucleic acids (4, 5). Hoffman, Schofield and Rich (5) described spin-labeling of amino acid attached to CCA terminal of tRNA to study helical characteristics of the aminoacyl end of the tRNA molecule. In our study, the 4-thiouridine residue located in the region between dihydrouridine stem and amino acid stem of *E. coli* tRNA's (Fig. 1) was chosen as a site for spin-labeling in order to investigate the conformational characteristics of the local region of the tRNA molecule. Specific spin-labeling of the 4-thiouridine residue of the tRNA's could be achieved under extremely mild conditions using N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)bromoacetamide (compound I) as spin-labeling reagent (Fig. 2). Our method provides a new approch to study of the conformational properties of tRNA's in relation to biological activities.

Materials and Methods: *E. coli* $tRNA_{II}^{Tyr}$, $tRNA^{fMet}$, $tRNA^{Val}$ and $tRNA^{Phe}$ were isolated as described previously (6-8). 4-Thiouridine 2',(3')-phosphate was synthesized as reported previously (9). N-(1-Oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)bromoacetamide synthesized by the method of Ogawa and McConnel (10) was supplied by Drs. Kitazono, Endo and Hamana of Kyushu University.

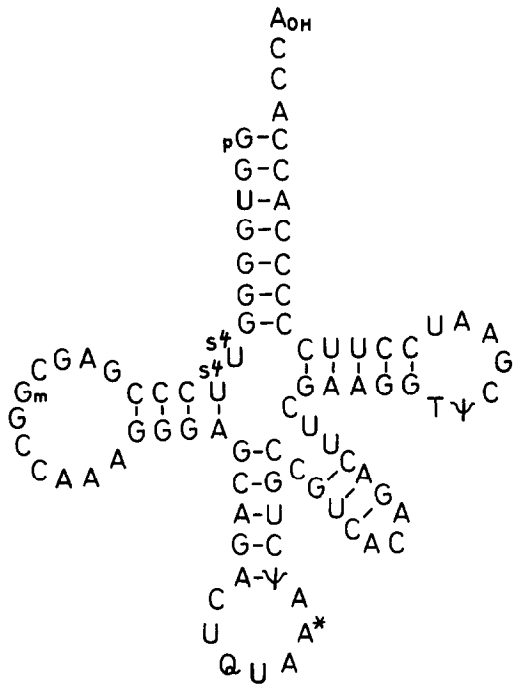
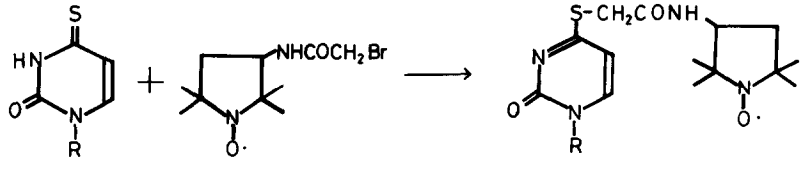


Fig. 1: Cloverleaf structure of E. coli $tRNA^{Tyr}_{II}$, indicating the location of 4-thiouridine (14, 15). In the sequences of E. coli $tRNA^{fMet}$ (7, 16), $tRNA^{Val}$ (2, 3) and $tRNA^{Phe}$ (17), the eighth position from the 5'-end was occupied by 4-thiouridine.



(I) (II)

Fig. 2: The spin-labeling of 4-thiouridylic acid. R=-β-D-ribofuranose 2',(3')-phosphate.

RNase T_1 was kindly provided by Dr. H. Okazaki of the Central Research Laboratories of Sankyo Co. Ltd. Pancreatic RNase (5 times recrystallized) was a product of Sigma Chemical Co.

Spin-labeling of 4-thiouridine 2',(3')-phosphate

4-Thiouridine 2',(3')-phosphate was treated with 5 fold excess of compound I in buffer containing 0.005 M Na_2CO_3 and 0.09 M $NaHCO_3$ (pH 8.9) at 20°C. On incubation for 30 min, the absorption maximum at 330 mμ characteristic of 4-thiouridylic acid completely disappeared, and two new maxima appeared at 270 mμ and 302 mμ (Fig. 3). Under the same incubation conditions, common

nucleotides such as 5'-AMP, 5'-GMP, 5'-UMP and 5'-CMP did not react at all with this spin-labeling reagent.

Spin-labeling of E. coli tRNA's

A sample of 200 O.D. units of a given tRNA was dissolved in 10 ml of buffer containing 0.005 M Na_2CO_3 and 0.09 M $NaHCO_3$ (pH 8.9). Then 11 mg of compound I, dissolved in 0.64 ml of methanol were added and the reaction mixture was incubated at 20°C with constant stirring. After incubation for 5 hrs, the absorbance at 330 mμ due to 4-thiouridylic acid residues had completely disappeared. The reaction mixture was then neutralized to pH 7.0 by adding 1 M sodium acetate buffer, pH 5.0. The spin-labeled tRNA was precipitated from the reaction mixture by addition of 2.5 volumes of cold ethanol. It was collected by centrifugation, dissolved in 2 ml of 1 M sodium acetate buffer, pH 5.0, and again precipitated by addition of ethanol. This precipitation procedure was repeated twice to remove excess reagent.

Detection and isolation of spin-labeled nucleotide from $tRNA_{II}^{Tyr}$

A sample of 100 O.D. units of spin-labeled $tRNA_{II}^{Tyr}$ was incubated with 50 μg of RNase T_1 in 2 ml of 0.05 M Tris-HCl buffer, pH 7.5 at 37°C for 18 hrs. The hydrolyzate was chromatographed on a column of DEAE-Sephadex A-25 with an NaCl gradient in the presence of 7 M urea at pH 7.5 as described previously (7) (Fig. 5). The chromatographic profile was identical with that of an RNase T_1 digest of control $tRNA_{II}^{Tyr}$, except that the oligonucleotide of peak 5 fraction, presumably $s^4U^*s^4U^*CCCG$ was eluted much faster than that of control untreated s^4Us^4UCCCG. After desalting, the electron paramagnetic

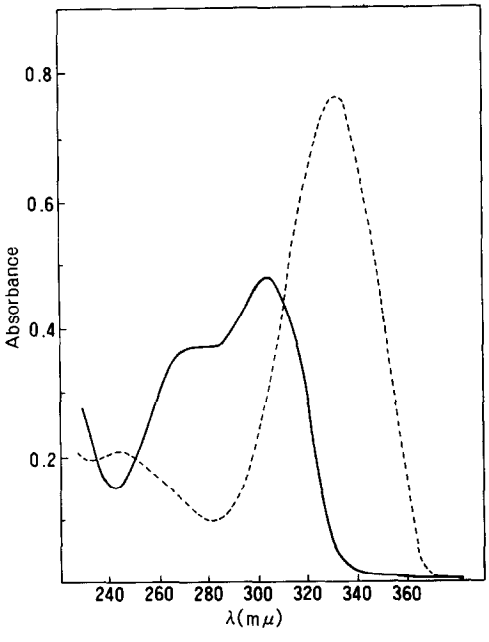


Fig. 3: UV absorption spectra of 4-thiouridylic acid and spin-labeled 4-thiouridylic acid. The spectra were measured in buffer containing 0.005 M Na_2CO_3 and 0.09 M $NaHCO_3$ (pH 8.9). ——— , 4-thiouridylic acid; - - - - - , spin-labeled 4-thiouridylic acid.

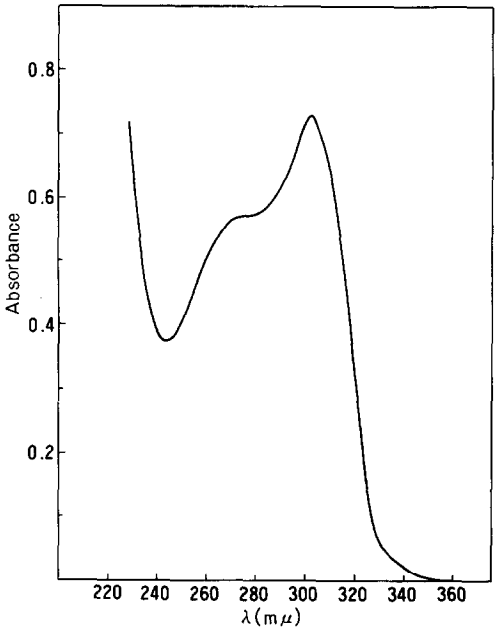


Fig. 4: UV absorption spectrum of spin-labeled 4-thiouridine 3'-phosphate isolated from $tRNA_{II}^{Tyr}$.

resonance (EPR) spectrum of each fraction was measured. Only peak 5 showed a nitroxide radical specific spectrum (Fig. 6c), all other peaks having no EPR absorption. The oligonucleotide in peak 5 ($s^4U^*s^4U^*CCCG$) was extensively digested with pancreatic RNase, and chromatographed on Toyo-Roshi, No 51A paper with ethanol-1 M ammonium acetate, pH 7.5 (7:3, v/v) as solvent. The spin-labeled nucleotide moved close to the solvent front, and was completely separated from other nucleotides such as Gp and Cp. It was eluted from the paper with distilled water and its UV absorption spectrum and EPR spectrum were found to be identical with those of spin-labeled 4-thiouridine 2',(3')-phosphate (Fig. 4, Fig. 6b).

Measurement of the EPR spectrum

The spin-labeled material was dissolved at a concentration of 40 to 60 O.D. units per ml in 0.05 ml of 0.2 M NaCl and 0.05 M Tris-HCl, pH 7.5. The EPR spectra of samples were measured in a Model JES 3BX ESR Instrument (Japan Electron Optics Laboratory Co.) operated at the X-band (9,400 kc) with 100 kc modulations.

Results and Discussion: 4-Thiouridine, first found in unfractionated E. coli tRNA by Lipsett (11) is known to be contained in $tRNA^{Tyr}$, $tRNA^{fMet}$, $tRNA^{Met}$, $tRNA^{Val}$, $tRNA^{Phe}$ and $tRNA^{His}$ from E. coli (12). We focused attention on the 4-thiouridine residue as a site for spin-labeling, since unlike other nucleotides it has selective reactivity (13). Compound I, N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)bromoacetamide was found to be a very selective reagent for spin-labeling of the 4-thiouridine residue in E. coli tRNA's.

Using 4-thiouridine 2',(3')-phosphate as a model, compound I reacted selectively with 4-thiouridylic acid to afford the spin-labeled 4-thiouridine derivative, compound II, as shown in Fig. 2. The reaction seems very specific, since four major nucleotides of RNA, AMP, GMP, UMP and CMP did not react under the same conditions. The extremely high selectivity of the reaction was confirmed using E. coli $tRNA^{Tyr}_{II}$. After hydrolysis of the spin-labeled $tRNA^{Tyr}_{II}$ with RNase T_1, spin-label was only found in the oligonucleotide fraction of s^4Us^4UCCCG. The spin-labeled derivative of 4-thiouridylic acid was recovered and characterized from its complete hydrolyzate by pancreatic RNase (Fig. 4, Fig. 6b). The experiment also indicated that minor components of $tRNA^{Tyr}_{II}$, i.e. ribothymidine, pseudouridine, 2'-O-methylguanosine, 2-methylthio-N^6-isopentenyladenosine and an unknown component designated as Q did not react

Abbreviations: s^4U, 4-thiouridine; s^4U^*, spin-labeled 4-thiouridine: Gm, 2'-O-methylguanosine; A*, 2-methylthio-N^6-isopentenyladenosine; Q, an unknown component corresponding to G* of Goodman et al. (15).

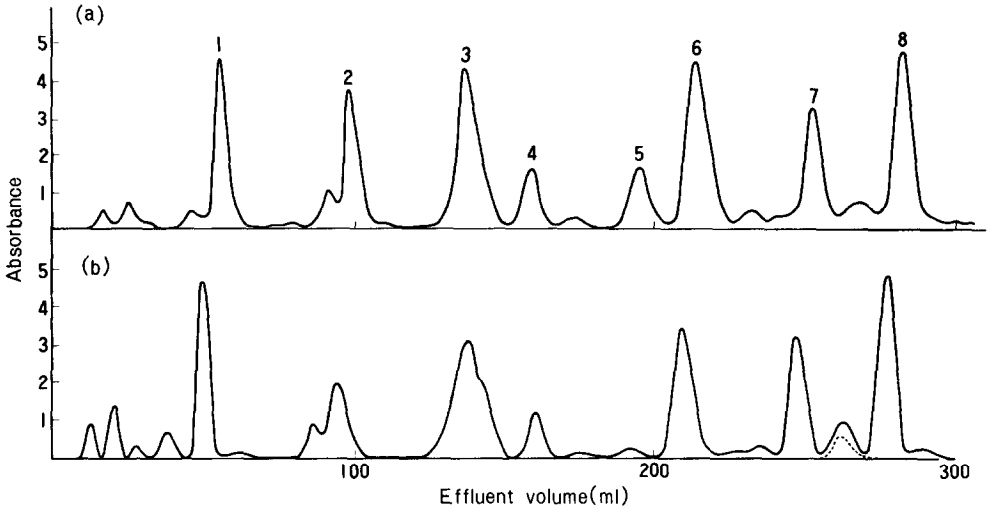


Fig. 5: Chromatographic separation of spin-labeled oligonucleotide s^4U^*-s^4U^*CCCG. a) Chromatography of an RNase T_1 digest of spin-labeled $tRNA^{Tyr}_{II}$. b) Chromatography of an RNase T_1 digest of control untreated $tRNA^{Tyr}_{II}$. ——, absorbance at 260 mμ; ------, absorbance at 330 mμ.

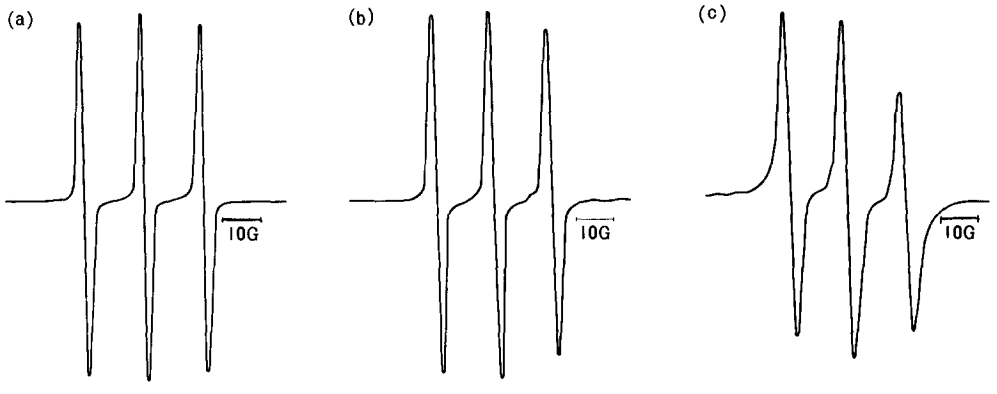


Fig. 6: EPR spectra of authentic compound II, spin-labeled nucleotide and oligonucleotide isolated from $tRNA^{Tyr}_{II}$. a) Authentic spin-labeled 4-thiouridylic acid. b) Spin-labeled nucleotide from $tRNA^{Tyr}_{II}$. c) Spin-labeled oligonucleotide, $s^4U^*s^4U^*CCCG$.

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with compound I (Fig. 1).

Spin-labeled 4-thiouridylic acid showed a completely symmetrical triplet spectrum (Fig. 6a). On the contrary, the spin-labeled oligonucleotide, $s^4U^*s^4U^*CCCG$ showed an asymmetrical spectrum, indicating that partial restric-

tion was pronounced at the oligonucleotide level, although the degree of restriction was much less than that observed in the spin-labeled tRNA (Fig. 6c).

Spin-labeling of the 4-thiouridylic acid residue of several E. coli tRNA's could be achieved in aqueous solution under very mild conditions. It should be mentioned that the amino acid acceptor activities of $tRNA^{Tyr}_{II}$, $tRNA^{fMet}$, $tRNA^{Val}$ and $tRNA^{Phe}$ were not affected by the modification, although some other tRNA's such as $tRNA^{Glu}$ and $tRNA^{Lys}$ lost considerable amino acid acceptor activities. It is possible that $tRNA^{Glu}$ and $tRNA^{Lys}$ contain minor components other than 4-thiouridine, which also react with the spin-labeling reagent. The EPR spectrum of spin-labeled tRNA's was much broader than that of spin-labeled 4-thiouridylic acid and that of spin-labeled oligonucleotide (Fig. 7). This indicates that free rotation of nitroxide radicals is fairly restricted in the intact tRNA molecule, suggesting that the 4-thiouridine region is rearranged so as to interact with some other part of the tRNA molecule. The

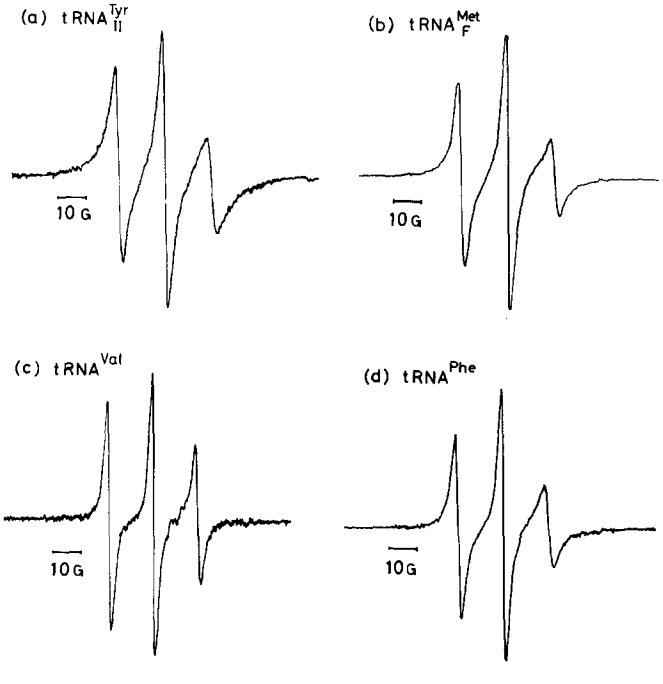

Fig. 7: EPR spectra of intact E. coli tRNA's a) $tRNA^{Tyr}_{II}$, b) $tRNA^{fMet}$, c) $tRNA^{Val}$, d) $tRNA^{Phe}$.

EPR spectra of $tRNA^{Tyr}_{II}$, $tRNA^{fMet}$, $tRNA^{Val}$ and $tRNA^{Phe}$ are not quite identical, indicating that the base interaction in the 4-thiouridine region differs in different tRNAs. This is of particular interest because 4-thiouridine is located in the same region in the primary sequences of all these tRNA's (1, 3, 7, 14-17) (Fig. 1). In this connection, it should be mentioned that the thermal melting profile at 330 mμ due to the 4-thiourydilic acid residue also differed in these tRNA's (12).

Thus specific spin-labeling of tRNA can be achieved by the present method. It is hoped that the availability of spin-labeled tRNA retaining biological activities may lead to an insight into the functional significance of the conformational characteristics of tRNA.

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References:

1. F. Harada, F. Kimura and S. Nishimura, Biochim. Biophys. Acta, 182, 590 (1969)
2. M. Yaniv and B. G. Barrell, Nature, 222, 278 (1969)
3. F. Harada, F. Kimura and S. Nishimura, Biochim. Biophys. Acta, in press
4. I. C. P. Smith and T. Yamane, Proc. Natl. Acad. Sci., U. S., 58, 884 (1967)
5. B. M. Hoffman, P. Schofield and A. Rich, Proc. Natl. Acad. Sci., U. S., 62, 1195 (1969)
6. S. Nishimura, F. Harada, U. Narushima and T. Seno, Biochim. Biophys. Acta, 142, 133 (1967)
7. T. Seno, M. Kobayashi and S. Nishimura, Biochim. Biophys. Acta, 169, 80 (1968)
8. K. Oda, F. Kimura, F. Harada and S. Nishimura, Biochim. Biophys. Acta, 179, 97 (1969)
9. M. Saneyoshi and F. Sawada, Chem. Pharm. Bull. (Tokyo), 17, 181 (1969)
10. S. Ogawa and H. M. McConnel, Proc. Natl. Acad. Sci., U. S., 58, 19 (1967)
11. M. N. Lipsett, J. Biol. Chem., 240, 3975 (1965)
12. T. Seno, M. Kobayashi and S. Nishimura, Biochim. Biophys. Acta, 174, 71 (1969)
13. M. Saneyoshi and S. Nishimura, Biochim. Biophys. Acta, 145, 208 (1967)
14. U. L. RajBhandary, S. H. Chang, H. J. Gross, F. Harada, F. Kimura and S. Nishimura, Fed. Proc., 28, 409 (1969)
15. H. M. Goodman, J. Abelson, A. Landy, S. Brenner and J. D. Smith, Nature, 217, 1019 (1968)
16. S. K. Dube, K. A. Marcker, B. F. C. Clark and S. Cory, Nature, 218, 232 (1968)
17. B. G. Barrell and F. Sanger, FEBS Letters, 3, 27 (1969)