RECONSTITUTION OF METHIONINE-ACCEPTOR ACTIVITY FROM FRAGMENTS OF *E. coli* tRNA^{fMet} WITH pCpGp DELETED FROM THE 5′-TERMINUS

T. SENO and K. SANO

Biology Division, National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo, 104 Japan

and

T. KATSURA

Faculty of Pharmaceutical Sciences, University of Tokyo, Hingo, 3-chome, Bunkyo-ku, Tokyo, Japan

Received 27 October 1970
Revised version received 17 November 1970

1. Introduction

In experiments on the reconstitution of methionine-acceptor activity from fragments of *Escherichia coli* tRNA^{fMet}, we have previously shown that cleavage of tRNA^{fMet} at the dihydrouridine-containing loop (D-loop) did not destroy the activity when the resulting 5′-quarter (fragment N) and 3′-three-quarter molecules (fragment L) were recombined [1]. Moreover, 40% of the original methionine-acceptor activity of the intact tRNA^{fMet} was retained after elimination of the nucleotide sequence in the D-loop. But removal of one further nucleotide, resulting in the loss of one base-pair in the stem region, diminished the activity [2]. These observations suggested that the stem part of the D-loop region may have an important role in maintaining the active form of the reconstituted molecules.

This paper reports that reconstituted molecules of *E. coli* tRNA^{fMet} with pCpGp deleted from the 5′-terminus, resulting in the loss of the first base-pair in the acceptor stem (see fig. 1a), can still accept methionine as well as formate with an efficiency of 50%. In contrast, Mirzabekov et al. have reported that in reconstituted yeast tRNA$_1^{Val}$ molecules, loss of the first two base-pairs in the acceptor stem, by the removal of pGpGp from the 5′-terminus (see fig. 1b), abolished the valine-acceptor activity [3]. These inconsistent results are discussed in relation to the different compositions of the base-pairs in the acceptor stems in the two tRNA species and also in relation to the hypothesis of Schulman and Chambers [4].

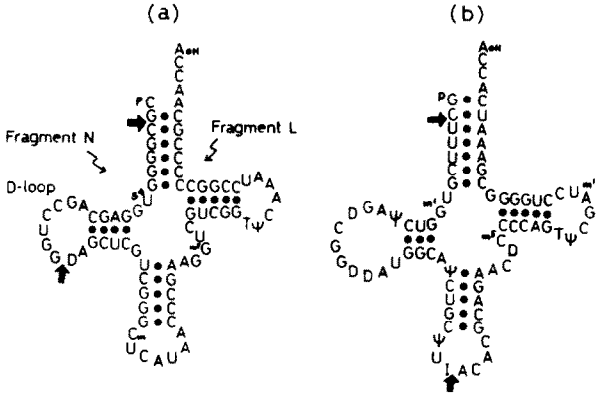

Fig. 1. Comparison of nucleotide sequence in the acceptor stem region of (a) *E. coli* tRNA^{fMet} [10] and (b) yeast tRNA$_1^{Val}$ [11]. The arrows indicate the sites of splitting discussed in the text. The U derivative in the original sequence of tRNA^{fMet} was replaced by 4 thiouridine [5] in (a).

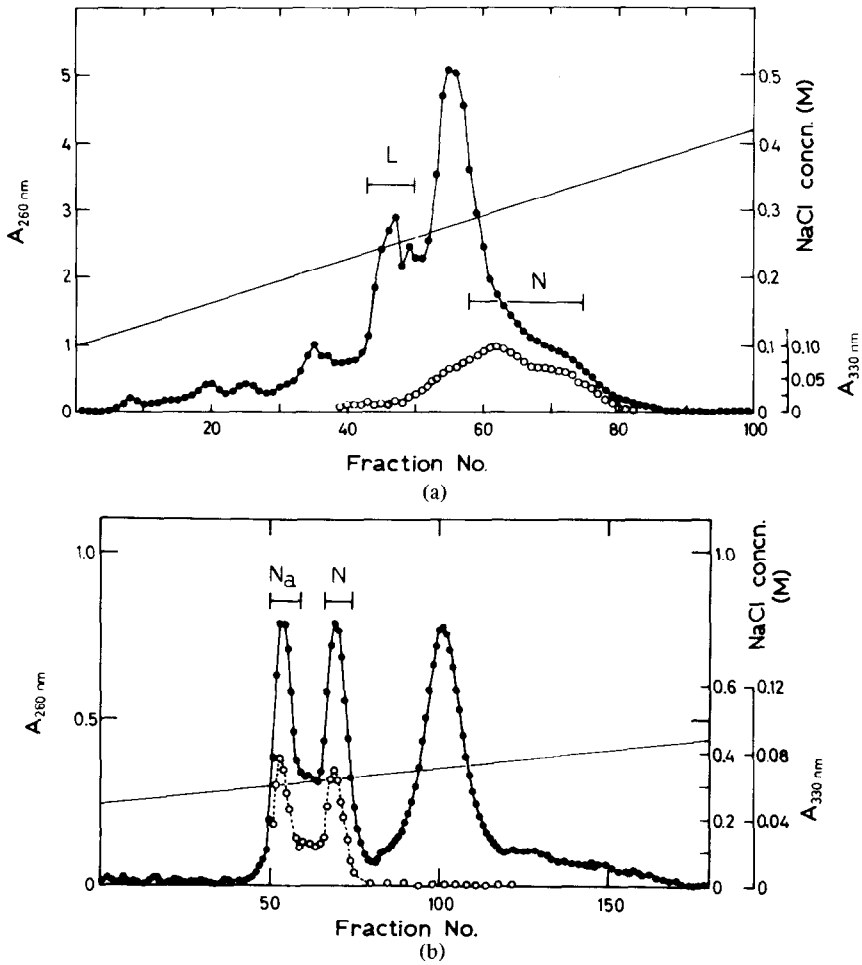

Fig. 2. Separation of fragments Na and N from *E. coli* $tRNA^{fMet}$ partially digested by ribonuclease T_1. (a) The first ion-exchange column chromatography at pH 2.7. (b) Further purification on N fractions from (a), by the ion-exchange chromatography at pH 7.5. Absorbance at 260 nm (•) and at 330 nm (○). For detailed procedures see text and [1].

2. Results

The $tRNA^{fMet}$ was purified from *E. coli* strain B as reported previously [5]. Limited digestion of $tRNA^{fMet}$ by ribonuclease T_1 was done at 37° in the presence of Mg^{2+} as described previously except that the incubation was for 1 hr instead of 26 min [1]. Fragment N and fragment L were isolated as reported previously [1] from the resulting oligonucleotide fragments. Because of the prolonged incubation with ribonuclease T_1, another 5′-quarter molecule (fragment Na) was also obtained, as is shown in fig. 2. The oligonucleotides were recovered from the two peaks as indicated in fig. 2b, and were subjected to finger-print analysis, after complete ribonuclease T_1 digestion, by two-dimensional thin-layer chromatography [6]. When the chromatograms were examined in UV light of 253.7 or 365.0 nm, the finger-prints from the two fragments were identical except that the pCpGp spot was absent in the digest from fragment Na (see fig. 3). This was confirmed in two separate experiments. From these results we concluded that fragment Na is shorter than fragment N by the deletion of pCpGp from the 5′-terminus (see fig. 1a).

Fig. 4 shows the extent of recovery of menthionine-acceptor activity of the L + Na complexes. When con-

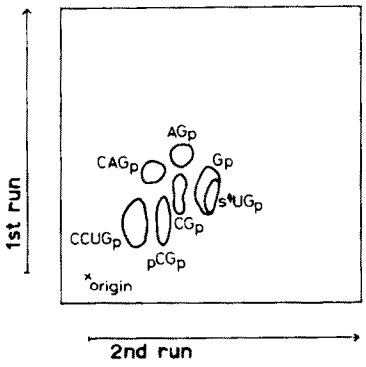

Fig. 3. Finger-print map of ribunuclease T_1 digest of fragment N. 0.5 A_{260} nm units of fragment N after hydrolysis by ribonuclease T_1 were applied to an Avicel SF cellulose glass plate (10 X 10 cm). First run in isobutyric acid–0.5 M NH_4OH (5 : 3, v/v) for 6 hr at 20°; second run in *tert.*-butanol–ammonium formate buffer pH 3.8 (1 : 1, v/v) [12] for 6 hr at 20°. The hydrolysate of fragment Na gave rise to an identical finger-print as that of fragment N except that the spot corresponding to pCpGp in the figure disappeared. The identity of each spot was established as described previously [6].

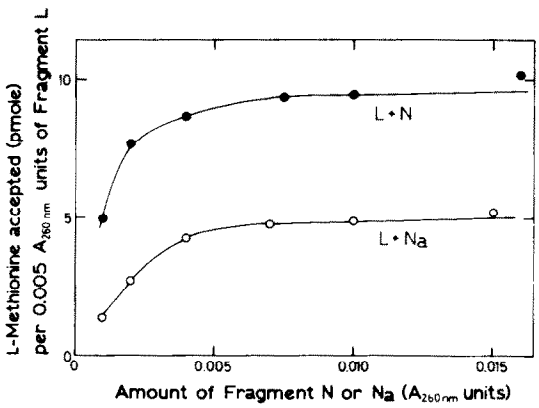

Fig. 4. Restoration of methionine-acceptor activity by the L + Na complexes. A constant amount of fragment L (0.005 A_{260} nm units) was mixed with increasing amounts of fragment Na in 0.01 M KCl, 0.01 M magnesium acetate and 0.05 M sodium cacodylate (pH 7.0) and pre-incubated at 50° for 15 min. The assay procedures for the incorporation of ^{14}C-methionine (specific activity, 187 mCi/mmole, a product of New England Nuclear Corp.) were as previously reported [1], except that the incubation was at 20° instead of 37° and that tris-HCl (pH 7.5) was replaced by sodium cacodylate (pH 7.0) in the reaction. The maximum level of methionine accepted by the L + N complexes in the figure (about 9.5 pmole) corresponded to the amount which 0.0045 A_{260} nm units of the intact $tRNA^{fMet}$ accepted under identical conditions.

stant amounts of fragment L were combined with increasing amounts of fragment Na, the restoration of methionine-acceptor activity reached about 50% of that obtained with the L + N complexes. Although the data are not shown, the L + Na complex could also accept formate. It should be mentioned that only about 30% recovery of the activity was obtained in a different preparation of fragment Na. Fig. 4 also shows that fragment Na was able to form a complex with fragment L as efficiently, physically, as did fragment N, since the amount of fragment Na required for the maximal recovery of activity was the same as that of fragment N. Preliminary data showed that the K_m value for methionyl-tRNA synthetase with L + Na complexes was ten times higher that with L + N complexes or intact tRNA (unpublished data). Hence it is most unlikely that the L + Na complexes are completely inactive and that observed recovery of activity is due to a 50% contamination of our fragment Na with fragment N. The finger-print analyses of the ribonuclease T_1 digests and the elution profiles of the two fragments (fig. 2b) do not suggest contamination of fragment Na.

It is concluded that the 5′-terminal sequence, pCpGp, of *E. coli* $tRNA^{fMet}$ molecule is not involved in the specificity sites for the recognition of either methionyl-tRNA synthetase or transformylase.

3. Discussion

In this study, a prolonged incubation of *E. coli* $tRNA^{fMet}$ with ribonuclease T_1 resulted in a removal of pCpGp from the 5′-terminus in addition to a cleavage at the D-loop. By combining this quarter-molecule with the 5′-end deleted (fragment Na) and the CCA-containing three-quarter molecule (fragment L), we have clearly demonstrated that the pCpGp sequence is not involved in the specificity site for methionyl-tRNA synthetase.

It is interesting to discuss the presence data in relation to the hypothesis proposed by Schulman and Chambers [4] concerning the location and the nature of the specific recognition site for aminoacyl-tRNA synthetase in the tRNA molecule. These authors proposed that the first three base-pairs in the acceptor

stem are required for specific recognition. Our present results are inconsistent with this hypothesis, since the removal of pCpGp from the 5′-terminus of the *E. coli* $tRNA^{fMet}$ molecule removed the first base-pair in the acceptor stem, but activity was not abolished. However, our present data do not rule out the possibility that the three nucleotides in positions 5, 6 and 7 from the 3′-terminus determine the specificity while the complementary nucleotides of the 5′-end are required to hold the linear sequence in the specific conformation required for recognition [7]. Contrary to the results presented here, Mirzabekov et al. have observed in the reconstitution of valine-acceptor activity from fragments of yeast $tRNA_1^{Val}$ that removal of pGpGp from the 5′-terminus abolished the activity [3].

The difference observed between these two tRNA species could be reasonably explained by the different composition of the base-pairs remaining in the acceptor stem after the removal of the dinucleotides from the 5′-terminus of each tRNA (see fig. 1). When pGpGp is removed from the 5′-end of yeast $tRNA_1^{Val}$, the remaining base–pairs in the acceptor stem are three A–U pairs followed by two G–C pairs, while in our L + Na complexes the remaining five base-pairs in the acceptor stem are all G–C (fig. 1). The G–C association is much more stable energetically than is the A–U one [8, 9]. Thus, the active conformation of the acceptor stem of yeast $tRNA_1^{Val}$ could no longer have been sustained by the tandem three A–U pairs. Another possible explanation of the difference between these two tRNA species could be that the specificity site for the aminoacyl-tRNA synthetase differs in tRNA species from, for example, yeast and *E. coli*; the hypothesis of Schulman and Chambers [4] may apply only to yeast tRNAs.

Our present results, when compared with those from yeast $tRNA_1^{Val}$, suggest that the retention of an ordered stem structure in the 5′-and 3′-terminal region is required for the reconstitution of amino acid-acceptor activity from fragments of tRNA molecules, and that elimination of only the first base-pair in the acceptor stem does not necessarily abolish activity.

Acknowledgements

We are grateful to Laboratories of Kaken Chemicals Ltd for the large-scale culture of *E. coli* strain B and isolation of crude *E. coli* tRNA. The authors are indebted to Susumu Nishimura for the supply of $tRNA^{fMet}$ and for critical discussions throughout this work. We also thank Takako Arima and Nobuko Shindo for their excellent technical assistance. This work was supported partly by research grants from the Japanese Ministry of Education.

References

[1] T. Seno, M. Kobayashi and S. Nishimura, Biochim. Biophys. Acta 190 (1969) 285.
[2] T. Seno, I. Kobayashi, M. Fukuhara and S. Nishimura, FEBS Letters 7 (1970) 343.
[3] A.D. Mirzabekov, D. Lastity, E.S. Levina and A.A. Bayev, Nature (1970) in press.
[4] L.H. Schulman and R.W. Chambers, Proc. Natl. Acad. Sci. U.S. 61 (1968) 308.
[5] T. Seno, M.Kobayashi and S.Nishimura, Biochim. Biophys. Acta 169 (1968) 80.
[6] F. Harada, F. Kimura and S. Nishimura, Biochim. Biophys. Acta 195 (1969) 590.
[7] R.W. Chambers, J. Cellular Physiol. 74, Suppl. 1 (1969) 179.
[8] Y. Kyogoku, R.C. Lord and A. Rich, Biochim. Biophys. Acta 179 (1969) 10.
[9] G. Felsenfeld and H. Todd Miles, Ann. Rev. Biochem. 36 (1967) 407.
[10] S.K. Dube, K.A. Marcker, B.F.C. Clark and S. Cory, Nature 218 (1968) 232.
S.K. Dube and K.A. Marcker, European J. Biochem. 8 (1969) 256.
[11] A.A. Bayev, T.V. Venkstern, A.D. Mirzabekov, A.I. Krutilina, L. Li and V.D. Axelrod, Molekul. Biol. 1 (1967) 754.
[12] G.W. Rushizky and C.A. Knight, Virology 11 (1960) 236.