KINETICAL STUDY ON THE RECONSTITUTION OF METHIONINE-ACCEPTOR ACTIVITY FROM FRAGMENTS OF *ESCHERICHIA COLI* $tRNA^{fMet}$ WITH A DELETION IN THE DIHYDROURIDINE-REGION OR THE AMINO ACID-ACCEPTOR STEM

T. SENO and K. SANO

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

Received 19 April 1971
Revised version received 3 June 1971

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-loop (D-loop) did not diminish the activity when the resulting 5′-quarter and 3′-three-quarter molecules were recombined [1]. Moreover, when the nucleotide sequence was eliminated from the D-loop [2] or from the acceptor stem [3], activity was lowered though it was still present. This paper describes kinetic studies on these defective complexes which showed that the lowered recovery of activity was not due to reduced ability of the fragments to recombine but to the reduced binding affinity to methionyl-tRNA synthetase of the complexes, as judged from the Michaelis constant. It was also shown that the subsequent aminoacylation step was not impaired with these defective complexes.

2. Results and discussion

Fig. 1 shows the saturation of methionine-acceptor activity by varying the mixing ratio of the complementary fragments from *E. coli* $tRNA^{fMet}$. The clover-leaf rearrangements expected with each combination of fragments are shown in figs. 2a and 2b. The characterizations of each fragment are described in the legend to fig. 2b. The results can be summarized as follows:

(a) The plateau level of recovery of activity on combination of Fragment La and N or Fragment L and Na was lower than that on combination of Fragment L and N. The mixing ratio of complementary fragments needed for the plateau level with the former combinations did not differ from that with the latter combination, in all cases being close to 3:1 measured from the ultraviolet absorbance, as expected from the chain lengths of the fragments (see fig. 2a and [1]).

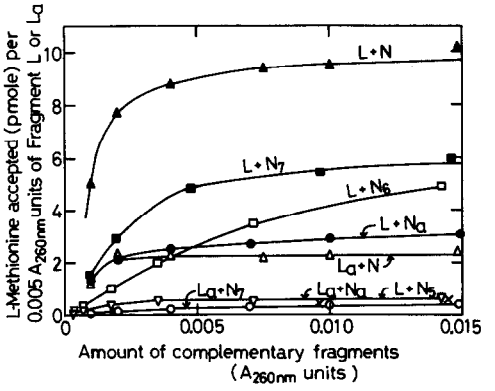

Fig. 1. Saturation of methionine-acceptor activity by varying the mixing ratio of complementary fragments from *E. coli* $tRNA^{fMet}$. A constant amount of Fragment L or Fragment La was mixed with increasing amounts of the complementary fragment specified in 50 μl of 0.01 M KCl, 0.01 M magnesium acetate and 0.05 M sodium cacodylate (pH 7.0) and preincubated at 50° for 15 min. Incorporation of ^{14}C methionine (specific activity, 187 mCi/mmole, product of New England Nuclear Corp.) was assayed as reported previously [3] after incubation at 20° for 15 min. The maximum level of methionine accepted by Complex L+N in the figure (about 9.5 pmole) corresponded to the amount accepted by 0.0045 $A_{260\,nm}$ units of intact $tRNA^{fMet}$ under identical conditions.

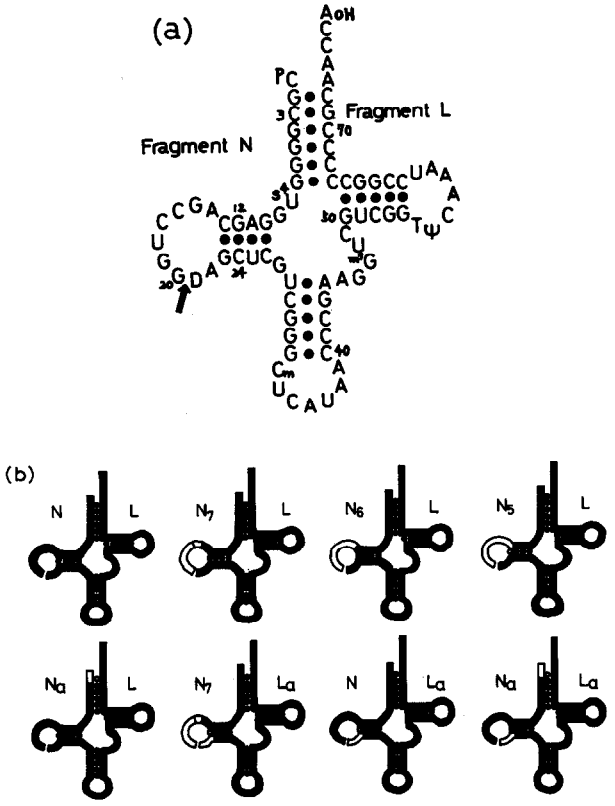

Fig. 2. (a) Nucleotide sequence of Fragment L (Residue 21–77) and Fragment N (Residue 1–19 or 20) obtained by a single cleavage of *E. coli* $tRNA^{fMet}$ at the D-loop. The arrow indicates the site of cleavage by ribonuclease T_1 (see [1]). The nucleotide residues were numbered in order from the 5′-terminus of the tRNA. The primary sequence and the arrangement in the clover-leaf pattern of the tRNA were taken from Dube et al. [4]. The U-derivative in the original sequence was replaced by 4-thiouridine [5]. (b) Schematic representation of clover-leaf rearrangements expected on combinations of the fragments of *E. coli* $tRNA^{fMet}$. Filled circles show base-pairs in the stem region. Open circles represent base-pairs lost as a result of nucleotide deletion. Fragment La (Residues 24–77) was obtained by further digestion of Fragment L by ribonuclease T_1 in the presence of Mg^{2+}. Chromatographic analysis showed that the nucleotide sequence of Fragment La was identical with that of Fragment L except that the DAG sequence at the 5′-end was lacking. The 5′-terminal nucleoside was confirmed to be cytidine by labeling the 5′-terminal nucleoside of the oligonucleotide with ^{32}P, as described in detail previously [1] (polynucleotide kinase and γ-^{32}P GTP were kindly given by Drs. M. Takanami [6] and M. Sugiura of Kyoto University). Fragment Na (Residues 3–19 or 20) has been characterized previously [3]. Fragment N_5 (Residues 1–13), Fragment N_6 (Residues 1–13) and Fragment N_7 (Residues 1–14 or 15) were described previously [2].

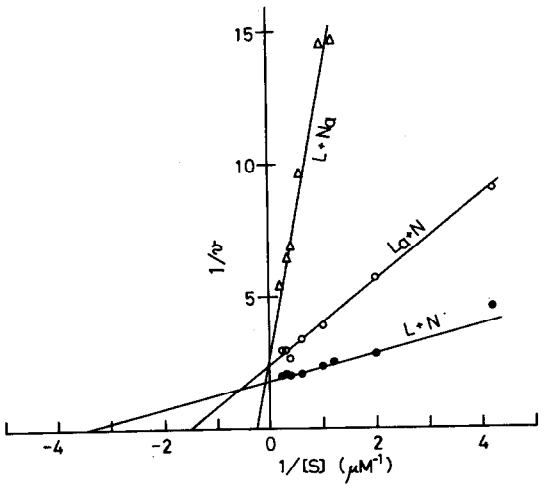

Fig. 3. Lineweaver-Burk plots for the acylations of Complexes L+Na, La+N and L+N. The complexes were prepared by mixing the complementary fragments in a ratio of 1:1 by ultraviolet absorbance so that the 5′-quarter molecule was in molar excess. The mixture was preincubated as described in the legend to fig. 1. The reaction mixture for assay of methionine-acceptor activity of the complexes contained the preincubated mixture of fragments specified, 430 pmoles of ^{14}C methionine (see legend to fig. 1) and 0.025 μg of partially purified *E. coli* methionyl-tRNA synthetase (prepared by a combination of the two-phase method [7], DEAE-cellulose chromatography and hydroxylapatite fractionation [8]). Other components of the reaction mixture were as described in the legend to fig. 1. Incubation was carried out at 15° for 4 min. ν: μmoles L-methionine incorporated per min per methionyl-tRNA synthetase fraction. S : Values were calculated assuming that 0.75 A_{260nm} units of the three-quarter molecule containing CCA (corresponding to 1 A_{260nm} unit of native $tRNA^{fMet}$, see fig. 2a) was equal to 1.66 nmoles of tRNA [9].

Thus, the low plateau level obtained with these combinations of fragments cannot be due to a reduced ability of the fragments to join together. On combination of Fragment L and N_6, no clear saturation plateau was obtained. This could be due to the impurity of Fragment N_6 with respect to the chain length of the oligonucleotides (see fig. 2 in [2]).

(b) On combination of Fragment La and Na, which resulted in deletion in both the D-loop region and the acceptor stem region, the recovery of activity was substantially zero. The specificity site for recognition of methionyl-tRNA synthetase may not be due to a single region of $tRNA^{fMet}$.

(c) The failure to recover activity on combination

of Fragment L and N_5 has previously been explained as due to a loss of the G–C pair in the D-stem [2]. The fact that no activity was recovered on combination of Fragment La and N_7 seems to favor this explanation. However, the considerable activity recovered on combination of Fragment La and N does not support this explanation, since one G–C pair is also absent in the identical position in the latter complex. This discrepancy could be explained by supposing that the sequence CUG (Residues 17–19) in the D-loop may incidentally form hydrogen-bonds with the sequence CAG (Residues 13–15) in the same fragment to form a loop structure.

Fig. 3 shows Lineweaver-Burk plots for acylation by Complex L+Na and Complex La+N using partially purified methionyl-tRNA synthetase. The apparent K_m for Complex L+Na obtained by extrapolating the plots was higher by one order of magnitude than that for Complex L+N. The apparent K_m for Complex La+N was about twice as high as that for Complex L+N. Complex L+N has been shown to be as active as intact $tRNA^{fMet}$ in terms of its K_m and v_{max} values [1]. The v_{max} values in fig. 3 do not show any significant difference between the three complexes. It seems that the binding affinity to methionyl-tRNA synthetase rather than the subsequent aminoacylation step was impaired by the conformation differences in Complex L+Na or Complex La+N.

In conclusion, the present study shows that partial deletion in the oligonucleotide fragments did not reduce the ability of the complementary fragments to form complexes but was sufficient to result in distortion in the necessary conformation of $tRNA^{fMet}$ for efficient binding to methionyl-tRNA synthetase. The subsequent acylation step did not seem to be impaired by these conformational changes.

Acknowledgements

We are grateful to members of the Laboratories of Kaken Chemicals Ltd. for large scale isolation of crude *E. coli* tRNA. The authors are indebted to Dr. S. Nishimura of this Institute for stimulating discussion and helpful criticism of our manuscript. Thanks are due to Dr. D. Söll of Yale University for valuable suggestions about preparing methionyl-tRNA synthetase from *E. coli*. This work was supported in part 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] T. Seno, K. Sano and T. Katsura, FEBS Letters 12 (1971) 137.
[4] S.K. Dube, K.A. Marcker, B.F.C. Clark and S. Cory, Nature 218 (1968) 232.
[5] T. Seno, M. Kobayashi and S. Nishimura, Biochim. Biophys. Acta 169 (1969) 80.
[6] M. Takanami, J. Mol. Biol. 23 (1967) 135.
[7] H. Hayashi, J.R. Knowles, J.R. Katze, J. Lapointe and D. Söll, J. Biol. Chem. 245 (1970) 1401.
[8] F. Lemoine, J.-P. Waller and R. van Rapenbusch, European J. Biochem. 4 (1968) 213.
[9] R.M. Hoskinson and H.G. Khorana, J. Biol. Chem. 240 (1965) 2129.