INABILITY OF sRNA DERIVED FROM PEPTIDYL sRNA TO ACCEPT AMINO ACIDS*

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It is well accepted that the growing polypeptide chain is linked to sRNA at the terminal end (Gilbert, 1963). It is assumed that the sRNA linked to polypeptide is the usual sRNA which accepts amino acid at its adenosine terminus (transfer RNA). In this communication we report that the sRNA derived from polyphenylalanyl sRNA does not accept phenylalanine. The isolation procedure for the sRNA from peptidyl sRNA is mild enough to recover most of the phenylalanine acceptor capacity of the usual sRNA (transfer RNA).

Materials and Methods - The system for polyphenylalanine formation was prepared from E. coli as previously described (Kaji et al., 1966). E. coli aminoacyl sRNA synthetase was prepared as previously described (Kaji et al., 1965). For the preparation of polyphenylalanyl sRNA, the reaction mixture contained the following in μmoles/0.9 ml: 126 Tris-HCl (pH 7.8), 12 magnesium acetate, 80 KCl, 6.3 β-mercaptoethanol, 3 ATP (potassium salt), 0.5 GTP and 6 phosphoenolpyruvate. In addition, it contained 10 μg of pyruvate kinase, 45 μc of C^{14}-phenylalanine (393 μc/μmole), 0.9 mg of E. coli sRNA, 0.36 mg of polyuridylic acid (poly-U), 0.5 mg of aminoacyl sRNA synthetase, and 1.35 mg of three times

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washed ribosomes. The mixture was incubated for 20 min at 22°C and 0.1 ml aliquot was mixed with 3 ml of a buffer containing 5×10^{-2} M KCl, 2×10^{-2} M magnesium acetate and 0.1 M Tris-HCl (pH 7.1)(Buffer 1). The mixture was then poured through a cellulose nitrate Millipore filter (pore size, 0.45 μ, 25 mm diameter) and the filter was washed with three 3 ml portions of Buffer 1. The bound phenylalanyl sRNA, polyphenylalanyl sRNA and ribosomes remained on the filter paper (Nirenberg & Leder, 1964). The phenylalanyl sRNA but not polyphenylalanyl sRNA was released from the ribosomes with a buffer contair 0.05 M KCl, 10^{-4} M magnesium acetate, 0.1 M Tris-HCl (pH 7.1)(Buffer 2). The polyphenylalanyl sRNA was released from ribosomes with 2 ml of 0.5% sodium dodecyl sulfate (lyophilized. The lyophilized powder which was pooled from the eluates of eight similar Millipore filter papers was mixed with 2 ml of 0.1 M sodium acetate buffer (pH 5.6). To th mixture were added 6 ml of ethanol and the precipitate which formed was dissolved in the 0.1 M Na-acetate buffer (pH 5.6). The polyphenylalanyl sRNA was again precipitated by the addition of 3 ml of ethanol. This was repeated three times to assure the removal of sod dodecyl sulfate. The polyphenylalanyl sRNA finally obtained was dissolved in 1 ml of 0.0 sodium acetate (pH 5.6) and kept at -20°C until used. The loss of radioactive material dur the isolation procedure was 37.5%. When a known amount of sRNA was subjected to the s procedure 92% of the sRNA was recovered as intact sRNA which could accept amino acid. ascertain whether the radioactive polyphenylalanine was still linked to sRNA after these procedures the sample was subjected to sucrose gradient centrifugation. As shown in Fig. 1 sedimentation of radioactive material of the sample was markedly different after the sample had been treated with RNase indicating that the radioactive peptide thus isolated was still linked to RNA.

Results – The data in Fig. 2A show the amino acid acceptor capacity of sRNA derived from polyphenylalanyl sRNA. In this experiment, polyphenylalanyl sRNA was incubated at pH 9.6. Aliquots were taken at the time intervals indicated and the acceptor

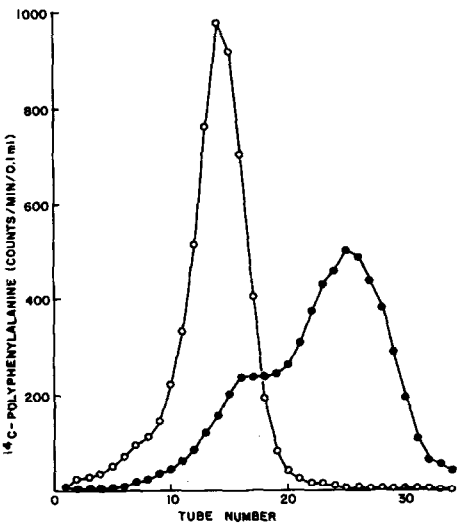

Fig. 1. Sedimentation Behaviour of Polyphenylalanyl sRNA. The polyphenylalanyl sRNA (0.07 ml containing 15,120 cpm) was incubated for 60 min at 30°C with 0.007 ml of water (o—o) or with 0.007 ml of a solution containing 35 µg of pancreatic RNase (●—●). After the incubation, 0.063 ml of water and 0.14 ml of 1% sodium dodecyl sulfate were added to the mixture. An aliquot (0.2 ml) was placed on top of 5 ml of sucrose gradient (5% → 20%) containing 0.5% sodium dodecyl sulfate, 0.1 M NaCl, and 0.001 M Tris-HCl (pH 7.1) and centrifuged 20 hrs at 15°C in a SW-50 Spinco rotor at a speed of 45,000 rpm. After the centrifugation, 4 drop-fractions were collected in test tubes by puncturing the bottom of the centrifuge tube. The test tubes were numbered from the bottom fraction to the top fraction. Of each fraction, 0.1 ml was used for measurement of radioactivity.

capacity for phenylalanine was measured. As a control, the usual sRNA preparation was subjected to an identical condition and the change of the acceptor capacity was measured. To assure that the control sRNA was subjected to the identical condition to which polyphenylalanyl sRNA was subjected, the former was incubated in the eluates from ribosomes. This eluate was prepared in exactly the same manner as the preparation of polyphenylalanyl sRNA except that the original reaction mixture contained no phenylalanine, ATP, GTP or their generator. As shown in Fig. 2B, only 20% of the acceptor capacity of the usual control sRNA was lost during 120 min incubation period. When the acceptor capacity of the sRNA from the polyphenylalanyl sRNA was tested only a negligible amount of radioactive phenylalanine was incorporated into sRNA even with the sample taken at 120 min after the onset of the deacylation reaction (Fig. 2A). To see how much deacylation of polyphenylalanyl sRNA was taking place during the incubation period, samples were taken at various time intervals from the deacylation mixture at pH 9.6, and subjected to sucrose density gradient centrifugation. The polyphenylalanine, being relatively small in molecular weight (average chain length was at most 17 residues, as described below) did not sediment as fast as polyphenylalanyl sRNA. Thus, one could measure how much deacylation took place by this technique. As

shown in Fig.2B, at 120 min after the onset of incubation, about 80% of polyphenylalanyl sRNA was deacylated.

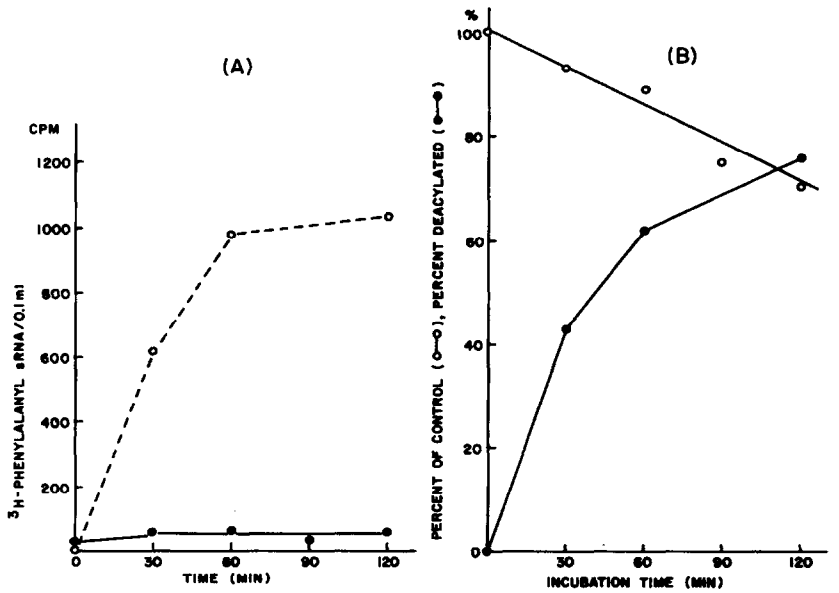

Fig.2(A). Phenylalanine acceptor capacity of sRNA derived from polyphenylalanyl sRNA. C^{14}-polyphenylalanyl sRNA (115,000 cpm in 0.3 ml) was mixed with 60 µl of 2 M Tr free base. The mixture (pH 9.6) was incubated at 30°C and 0.06 ml aliquot was taken at the time intervals and mixed with 0.5 µl of glacial acetic acid to lower the pH to about neutralit The volume of the mixture was adjusted to 0.1 ml with Buffer 2. To this solution was added 0.035 ml of a sRNA assay mixture which contained the following in µmoles/1.4 ml: 500 Tris-HCl (pH 7.8); 24 magnesium acetate; 20 mercaptoethanol; 10 ATP; 20 phosphoenolpyruvate. In addition, it contained 100 µg of pyruvate kinase, 20 µg of aminoacyl sRNA synthetase and 0.5 µc of ^{3}H-phenylalanine. The mixture was incubated for 15 min at 30°C and aliquot (0.1 was treated with cold (5°C) 5% trichloracetic acid and the radioactive phenylalanyl sRNA formed per 0.1 ml of the reaction mixture is plotted against the time of incubation for de-acylation of polyphenylalanyl sRNA at pH 9.6. •—•, sRNA from polyphenylalanyl sRNA; o---o, Theoretical value of what one should expect if sRNA from polyphenylalanyl sRNA can accept phenylalanine. These values were calculated on the basis of radioactivity of polyphenylalanyl sRNA, average chain length of polyphenylalanine as shown in Table 1, the percentage of deacylation of polyphenylalanyl sRNA measured by the sucrose gradient centrifugation technique and the percentage loss of phenylalanine acceptor capacity of control sRNA during the deacylation reaction.

(B) Effect of incubation at pH 9.6 on the phenylalanine acceptor capacity of sRNA and the time course of deacylation of polyphenylalanyl sRNA. o—o, time course of loss of phenylalanine acceptor capacity of sRNA upon incubation at pH 9.6. E. coli sRNA (0.3 mg) was suspended in 0.3 ml of a "ribosomal eluate". The "ribosomal eluate" was prepared in an identical fashion to the preparation of polyphenylalanyl sRNA except that the phenylalanine, ATP, GTP, and their generator were not added to the original reaction mixture for poly-phenylalanine formation. The sRNA solution (0.3 ml) was incubated and ^{3}H-phenylalanine acceptor capacity was measured in an identical manner to (A). The data were plotted as

percentage of the control value (1951 cpm of ^{3}H-phenylalanyl sRNA formed before incubation at pH 9.6). •——•, time course of deacylation of polyphenylalanyl sRNA. Polyphenylalanyl sRNA (34,500 cpm/0.09 ml) was mixed with 0.09 ml of water, and 0.018 ml of 2 M Tris (free base) and the mixture was incubated at 30°C. Aliquots (0.06 ml) were taken at the time intervals, the pH was adjusted to neutrality with glacial acetic acid, 0.12 ml of 1% sodium dodecyl sulfate was added, and the final total volume was adjusted to 0.24 ml with water. Of this mixture, 0.2 ml was used for sucrose density gradient centrifugation as described in Fig. 1. Percentage of deacylation was calculated from the radioactivity found at the top portion of the tube after the centrifugation.

To determine how much [^{3}H] -phenylalanine acceptor capacity one could expect from the sRNA of polyphenylalanyl sRNA, it was necessary to calculate the amount of polyphenylalanyl sRNA in the preparation. Thus, polyphenylalanyl sRNA was treated with dinitrofluorobenzene as described previously (Momose & Kaji, 1966) and the radioactivity at the amino-terminal of the chain and that of the internal chain was measured (Table 1). In four different experiments, the average chain length of polyphenylalanine calculated from these data ranged from 4.5 to 17.1 phenylalanine residues. On the basis of these data, total radioactivity of C^{14}-polyphenylalanyl sRNA and the degree of deacylation of polyphenylalanyl sRNA one could calculate the theoretical value of [^{3}H] -phenylalanyl sRNA which should be formed if the sRNA from polyphenylalanyl sRNA could accept [^{3}H] -phenylalanine. Such theoretical values were obtained and plotted against the time of incubation for deacylation. As shown in Fig. 2A (o---o) the expected value was at least 10-fold higher than the observed value.

Table 2 shows the results of calculations based on the data obtained in Table 1. From the radioactivity corresponding to dinitrophenyl phenylalanine one could calculate how much sRNA should be present in the peptidyl sRNA preparation (column 1). It was also possible to compute how much sRNA should be present from the total radioactivity of polyphenylalanyl sRNA and the average chain length (column 2). If these values are compared with the actual amount of sRNA specific for phenylalanine present in the sample, it is clear that the actual values are much less than the expected value. These data support the notion that sRNA from polyphenylalanyl sRNA does not accept phenylalanine.

TABLE 1

Chain length determination of polyphenylalanine

Exp.	C^{14}-polyphenylalanyl sRNA used (cpm)	Radioactivity in aqueous phase (cpm)	Radioactivity in ether phase (cpm)	Average numb of phenyl-alanine/chai
1	20,500	11,450	2,970	4.9
2	16,550	8,440	2,420	4.5
3	191,800	129,500	8,500	16.2
4	108,150	84,300	5,220	17.1

Peptidyl sRNA in 0.5 ml 0.01 M Na-acetate buffer (pH 5.6) was mixed with 0.5 ml 10% $NaHCO_3$ and 2 ml of 5% dinitrofluorobenzene in ethanol. The mixture was shaken at ro temperature for 15 hrs. After dinitrophenylation, 0.2 ml of 6 N HCl was added and the mixt was extracted with four 5 ml portions of ether pretreated with $FeSO_4$. Some radioactivity, possibly dinitrophenyl C^{14}-diphenylalanine representing 15-25% of total radioactivity was fc in this ether fraction. To the aqueous phase was added 0.5 ml of 12 N HCl and dinitropheny polyphenylalanine was hydrolyzed by incubating at 108°C for 18 hrs under N_2 atmosphere. A the hydrolysis dinitrophenyl phenylalanine was extracted with four 2 ml portions of ether.

TABLE 2

Comparison of observed phenylalanine acceptor capacity of sRNA, derived from polyphenylalanyl sRNA with theoretical capacity

Exp.	(1) μμmoles of NH_2 terminal	(2) μμmoles of sRNA calculated from chain length	(3) μμmoles of phenyl-alanine accepted	% (3)/(1)	(3)/(2)
1	7.57	10.67	1.16	15.3	10.9
2	6.15	9.38	0.80	13.1	8.6
3	21.65	30.12	0.75	3.5	2.5
4	13.25	14.70	0.33	2.5	2.2

The values were calculated from the observed radioactivity as described in Table 1. The phenylalanine acceptor capacity was measured as in Fig.2. The values in column 2 were calculated from the total radioactivity of polyphenylalanyl sRNA and the average chain length of each preparation of polyphenylalanyl sRNA.

Discussion - It is surprising that sRNA from polyphenylalanyl sRNA does not accept phenylalanine. Further tests showed that this sRNA did not accept other amino acids such as leucine, methionine, lysine, or tyrosine. There appear to be two possible explanations for this observation. The first explanation is that the sRNA in the polyphenylalanyl sRNA is destroyed during the isolation procedure. For this explanation, it is assumed that the sRNA of

polyphenylalanyl sRNA is much more labile than the sRNA in its free state because usual sRNA preparation loses only 20% of its phenylalanine acceptor capacity during the isolation procedure. This assumption is not entirely unreasonable considering the fact that polyphenylalanyl sRNA has stronger affinity to 50 S ribosomal subunits than aminoacyl sRNA. If the linking of sRNA with polyphenylalanine changes the configuration of sRNA in such a way that it can bind more tightly to 50 S ribosomal subunits, it is reasonable to assume that it also changes its configuration so that it becomes more labile.

The other possible explanation is that sRNA of the polyphenylalanyl sRNA is different from usual sRNA which accepts amino acid. For this explanation it is postulated that there exists a kind of sRNA which holds growing peptide but does not accept amino acid. In this discussion this hypothetical RNA is tentatively called holding RNA (hRNA). If hRNA exists, it should have similar sedimentation behaviour to that of sRNA. Since polylysine was reported to be linked to sRNA through adenosine (Bretcher, 1963), hRNA must have a terminal adenosine group. In this connection, it is interesting to speculate that 5 S sRNA (Rosset & Monier, 1963; Rosett, Monier, Julien, 1964; Schleich & Goldstein, 1966) may be hRNA. Experiments are in progress to determine which of the above two possible explanations is correct.

Summary - sRNA isolated from polyphenylalanyl sRNA does not accept phenylalanine. The isolation procedure is mild enough to recover most of the usual sRNA. This observation suggests two possibilities: 1) sRNA becomes more labile when linked to polyphenylalanine or 2) sRNA in polyphenylalanyl sRNA is different from the usual sRNA which accepts amino acids.

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