THE INACTIVATION AND REACTIVATION OF RIBOSOMAL-PEPTIDYL TRANSFERASE OF E. COLI

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INTRODUCTION

Peptidyl transferase, the enzyme responsible for peptide bond formation, appears to be located on the ribosome (Traut and Monro, 1964; Rychlik, 1966; Zamir et al., 1966), specifically on the 50S subunit (Monro, 1967; Maden et al., 1968). It is conveniently assayed with the "fragment reaction", in which 70S or 50S ribosomes catalyze the formation of Fmet-puromycin* from puromycin and Fmet-hexanucleotide (FM-T_1), the 3' terminal fragment produced by RNase T_1 digestion of Fmet-$tRNA_F$. The fragment reaction takes place in a medium containing 30% ethanol and at 0°C (Monro and Marcker, 1967).

While studying this reaction we became aware, that ribosomes prepared in different ways varied considerably in their ability to catalyze the fragment reaction. In some cases activity was completely absent. Nevertheless, the inactive ribosome preparations were active in other reactions in which peptide synthesis took place e.g. the poly U-directed polymerization of phe with added transfer enzymes, or the ApUpG-directed puromycin reaction of Fmet-tRNA in the presence of initiation factors (Vogel et al., 1967).

The loss of peptidyl-transferase activity was first attributed to a removal of some essential protein factor, during the purification of the ribosomes, which was added back with the transfer or initiation factor preparations. However, attempts to restore peptidyl transferase activity by adding these factors, were unsuccessful. Further investigation has shown, that inactivation of ribosomal-peptidyl transferase

* Abbreviations: Fmet - N-Formylmethionine; phe - phenylalanine; DTT - dithiothreitol; EDTA - ethylenediamine tetraacetate.

is due to the removal of NH_4^+ ions during the purification of the ribosomes. The readdition of NH_4^+ restores activity, provided that the ribosomes are also heated. The necessary conditions for reactivation are provided in reactions assayed at 30° or 37° (e.g. polymerization of phe) but not in reactions that take place at 0°, (e.g. the fragment reaction). The results suggest that the peptidyl transferase enzyme, or the ribosome in which it is located, can exist in two different conformations, only one of which is active.

METHODS

Ribosomes were prepared from E. coli MRE 600 (Cammack and Wade, 1965). Unfractionated tRNA was charged with C^{14}-met (New England Nuclear, specific activity 218) and enzymatically formylated according to Vogel et al. (1968). Digestion of Fmet-tRNA with RNase T_1, isolation of FM-T_1 and the fragment reaction were carried out essentially as described by Monro and Marcker (1967), except that the reaction mixture for the fragment reaction also contained 0.04 M NH_4Cl. Each reaction mixture (in 100 µL) contained a rate limiting amount of ribosomes. Incubation was at 0°C for 15 minutes. The reaction was assayed by two different methods: (a) 0.5 ml of 2% potassium acetate, at pH 5.5, in 90% ethanol was added to terminate the reaction, and the mixture was centrifuged at 2000 rpm for 10 minutes. An aliquot of the supernatant was removed and counted. (b) The reaction was terminated by the addition of 1.5 ml 0.1 M Tris-HCl pH 9.0, and the mixture was extracted twice with 1.5 ml portions of ethylacetate (Leder and Bursztyn, 1966). These extracts were combined and an aliquot was removed and counted.

RESULTS

a. Inactivation of ribosomal-peptidyl-transferase activity

The ribosomes employed in the study of the fragment reaction, were prepared by two different methods, that of Nishizuka and Lipmann (1966) and that of Kurland (1966). Both preparations were active in catalyzing the poly U-directed polymerization of phe. Ribosomes prepared according to the first method were highly active in the fragment reaction, whereas ribosomes prepared by Kurland's method had little or no activity. On examining the individual steps of the Kurland purification it was found, that inactivation occurred during the step in which ribosomes were dialyzed against 10 mM Tris-succinate pH 8.0, 10 mM $MgCl_2$ (TSM buffer). The active ribosomes (prepared according to Nishizuka and Lipmann) are not exposed in the course of their purification to a medium lacking NH_4. However, when

TABLE 1. Effect of dialysis against various monovalent cations on the subsequent activity of ribosomes in the fragment reaction

	Activity in fragment reaction[3]					
	Specific activity[4] (μμmoles of Fmet-puromycin formed per mg ribosomes)			Relative specific activity (% of control)		
Ribosomes[1]	a	b	c	a	b	c
Treatment[2]						
No dialysis	9.7	5.5	12.1	100	100	100
Dialysis against :						
TSM	0.1	0.2	0.0	1	4	0
TSM+0.05 M NH_4Cl	7.9	3.0	16.8	81	55	138
TSM+0.05 M KCl	6.3	2.1	6.0	65	38	49
TSM+0.05 M LiCl	1.3	0.2	0.0	13	4	0
TSM+0.05 M NaCl	1.3	0.4	0.3	13	7	2

(1) Ribosomes preparations were : a. according to Nishizuka and Lipmann (1966). b. Ribosomes were purified by two cycles of high speed centrifugation and suspension in 20 mM Tris-HCl at pH 7.4, 2 mM Mg $(OAc)_2$, 1 M NH_4Cl, 1 mM EDTA and 1 mM DTT. The last wash cycle and the final suspension of ribosomes were in TSM containing 100 mM NH_4Cl, 1 mM DTT and 1 mM EDTA. c. Isolated 50S subunits were prepared by differential centrifugation (Atsmon, 1968).

(2) Dialyses were carried out for 20 hours against a total buffer volume of 3 l, with two changes. The buffers included in addition to the indicated salts at 50 mM, also TSM and 0.2 mM DTT.

(3) The assay of the fragment reaction was described under METHODS. The amount of ribosomes per reaction mixture was 300 μg for preparation a and 150 μg for b and c. The reaction mixtures for ribosome preparations a and c contained 5000 cpm (12 μμmoles) of FM-T_1 and the assay was by ethanol precipitation. The mixture for b contained 3000 cpm (7 μμmoles) of FM-T_1 and the assay was by ethylacetate extraction (see METHODS).

(4) All the values represent net increase due to puromycin. Values of controls minus puromycin were substracted.

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subjected to dialysis against TSM, they too became unable to catalyze the fragment reaction. Some illustrative results are summarized in Table 1. Regardless of the initial steps employed in their purification, all the types of ribosomes tested undergo inactivation when dialyzed against TSM buffer. The addition of

NH_4Cl to the dialysis buffer largely prevents the loss of activity. Table 1 also shows the effect of dialysis against monovalent cations other than NH_4^+: Li^+, Na^+ and K^+ (all as chlorides). Of these, only K^+ can replace NH_4^+ although, at the concentration tested, less efficiently. All these effects are also observed with a preparation of isolated 50S subunits, indicating that inactivation does not involve the interaction of 30S and 50S subunits.

b. Reactivation of ribosomal-peptidyl-transferase activity

As was indicated before, the ribosomes, which were inactive in the fragment reaction, did function in other reactions in which peptide bond formation took place (e.g. polymerization of phe). Therefore, we had to assume that enzymatic activity was restored to the ribosomes under conditions in which polymerization was tested. Of the many differences between the conditions of the fragment and polymerization reactions the critical one was found to be the temperature at which the reactions were tested : the fragment reaction at 0°, and polymerization at 37°. Thus, peptidyl transferase was reactivated when the ribosomes were preheated. The heating medium had to contain NH_4^+ or K^+ ions. In their absence no heat reactivation was observed. The addition of monovalent cations to inactive ribosomes preincubated at $0^\circ C$ was ineffective. The rate of reactivation

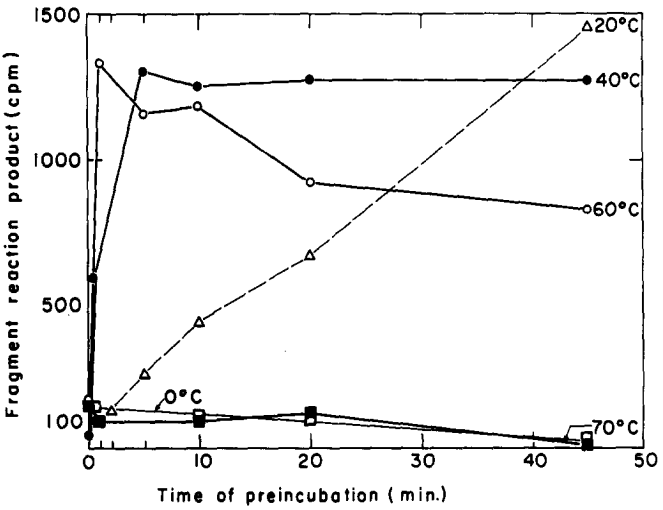

FIGURE 1. The rate of ribosomal-peptidyl-transferase reactivation at different temperatures. The ribosomes tested were prepared according to Kurland (1966). They were preincubated at a concentration of 7.5 mg/mL at the indicated temperatures in a buffer containing 5 mM Tris-succinate pH 8.0, 5 mM $MgCl_2$, 50 mM NH_4Cl. The assay of the fragment reaction was by ethanol precipitation as described in Table 1. Each reaction mixture contained 300 µg of ribosomes.

TABLE 2. Effect of preincubation of ribosomes with various monovalent cations on the activity of ribosomal-peptidyl-transferase

Ribosome treatment	Activity in fragment reaction	
	Specific activity (μμmoles of Fmet-puromycin formed per mg of ribosomes)	Relative specific activity (% of control)
preincubation with 0.05 M NH_4Cl	9.3	100
" " 0.05 M KCl	6.4	69
" " 0.05 M LiCl	1.5	16
" " 0.05 M NaCl	0.0	0
No added monovalent cation	0.7	8
preincubation with 0.05 M NH_4Cl and 0.05 M LiCl	9.5	102
preincubation with 0.05 M NH_4Cl and 0.05 M NaCl	8.2	88

The ribosomes were as in Figure 1. Preincubation was at 40°C for 5 minutes, in a buffer containing 5 mM Tris-succinate pH 8.0, 5 mM $MgCl_2$ with the indicated salts added. The fragment reaction was assayed as in Figure 1.

depended strongly on temperature as is shown in Fig. 1. The experiment was performed as follows: Inactive ribosomes were incubated with NH_4Cl at different temperatures, and aliquots were removed at intervals, rapidly chilled to 0°C (where no further reactivation takes place) and subsequently assayed in the fragment reaction at the standard temperature of 0°C. Reactivation occurred at higher temperatures: slowly at 20°C, and rapidly at 40° and 60°C. At the latter temperature the ribosomes were slowly inactivated by heat, and at 70°C the inactivation was rapid and complete.

The effectiveness of different monovalent cations in promoting reactivation is shown in Table 2. The specificity is similar to that observed in the dialysis experiments shown in Table 1. NH_4^+ ion is the most effective, K^+ is less effective, and Li^+ or Na^+ are no more effective than a medium lacking alkali metal ions. When preincubation with NH_4^+ was performed in the presence of Na^+ or Li^+, the effect was similar to that of NH_4 alone. That is, neither Na^+ nor Li^+ inhibited the reactivation. In these cases Li^+ or Na^+ were also present in the fragment reaction mixture at a concentration of 20 mM. This did not have any significant effect on the reaction. As with inactivation, the reactivation process did not depend on the presence of 30S

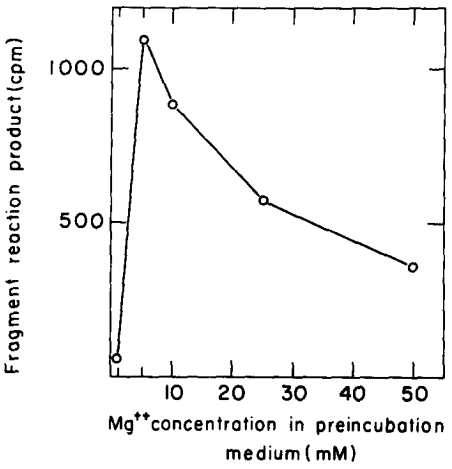

FIGURE 2. The effect of Mg^{++} concentration in the preincubation medium on the rate of reactivation of ribosomal-peptidyl-transferase. All the conditions were as in Table 2. The preincubation buffer contained 5 mM Tris-succinate pH 8.0, 50 mM NH_4Cl and the indicated concentrations of $Mg(OAc)_2$.

subunits, and 50S subunits gave essentially the same results as 70S ribosomes.

The above reactivation experiments were carried out at 5 mM Mg^{++}. Figure 2 shows the effect of Mg^{++} concentration on the rate of reactivation. The rate is markedly dependent on $[Mg^{++}]$ with a sharp optimum at 5 mM.

DISCUSSION AND SUMMARY

It has been shown in the above experiments that peptidyl-transferase, whose activity was studied either with isolated 50S particles or in the 70S complex, may undergo extreme changes in reactivity under conditions, which are commonly employed to purify and assay ribosomes. The fragment reaction is particularly useful in detecting these changes, since no change in reactivity occurs under the specific conditions of this reaction.

The active form of ribosomal-peptidyl transferase is dependent on the presence of NH_4^+ or K^+ ions. Peptidyl transferase activity is lost if these ions are removed and is restored when added back, but only if the ribosomes are also heated. The dependence of the reactivation process on heating is of particular interest in view of the finding of Traub and Nomura (1968), that heating is required for the reconstitution of biologically-active 30S particles. Maden et al. (1968) have recently reported, that exposure of polyphe-charged ribosomes to media lacking monovalent cations inhibited the rate of the subsequent puromycin reaction. In another, unrelated experiment they

have observed a slight stimulation in the rate of the puromycin reaction at 0°C, after having preincubated the polyphe-charged ribosomes at 30°C. Our results indicate that these phenomena are correlated.

It appears that 50S particles may exist in,at least, two different forms, only one of which has peptidyl transferase activity. The conditions under which one form is converted into the other suggest, that some structural rearrangement is involved in the transition. Whether the peptidyl transferase enzyme alone or a larger part of the ribosome is involved cannot be said at present. The transition, however, is not accompanied by any measurable change in the sedimentation constant of the 50S particle (Miskin, 1968).

Since these transitions take place under relatively mild conditions,the possibility may be entertained, that similar changes are involved in the process of normal protein synthesis. It is also possible, that such transitions may be brought about in the cell in a different way than that described in the present experiments.

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REFERENCES

Atsmon, A., Ph.D. Thesis, The Weizmann Institute (1968).
Cammack, V. A. and Wade, H. E., Biochem. J., 96, 621 (1965).
Kurland, C. G., J. Mol. Biol., 18, 90 (1966).
Leder, P. and Bursztyn, H., Biochem. Biophys. Res. Comm., 25, 233 (1966).
Maden, B. E. H., Traut, R. R. and Monro, R. E., J. Mol. Biol., 35, 333 (1968).
Miskin, R., Unpublished results (1968).
Monro, R. E., J. Mol. Biol., 26, 147 (1967).
Monro, R. E. and Marcker, K. A., J. Mol. Biol., 25, 347 (1967).
Nishizuka, Y. and Lipmann, F., Proc. Natl. Acad. Sci., 55, 212 (1966).
Rychlik, I., Biochim. Biophys. Acta, 114, 425 (1966).
Traub, P. and Nomura, M., Proc. Natl. Acad. Sci., 59, 777 (1968).
Traut, R. R. and Monro, R. E., J. Mol. Biol., 10, 63 (1964).
Vogel, Z., Zamir, A. and Elson, D., Israel J. Chem., 5, 114p (1967).
Vogel, Z., Zamir, A. and Elson, D., Proc. Natl. Acad. Sci., in press (1968).
Zamir, A., Leder, P. and Elson, D., Proc. Natl. Acad. Sci., 56, 1795 (1966).