Translocase Activity in the Aminoacyl Transferase II Fraction from Rat Liver

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Aminoacyl transfer from aminoacyl sRNA to ribosome-bound peptidyl sRNA in the cell-free rat liver system requires GTP, Mg^{++}, a monovalent ion (K^+ or NH_4^+), a reduced sulfhydryl compound and at least two soluble protein fractions, T1 and T2[2] (Gasior and Moldave, 1965; Moldave, 1965; Schweet and Heintz, 1966). This study utilized the reaction of ^{3}H-puromycin with the growing polypeptide chain to differentiate between the processes of peptide bond formation per se and translocation, i.e., the transfer of the newly lengthened polypeptide chain back to a site from which it can again form a peptide bond (Traut and Monro, 1964). Rat liver ribosomes are well suited for these studies since they are easily prepared with their natural nascent polypeptide chains intact. The reaction of ^{3}H-puromycin with the polypeptide chain to form peptidyl-^{3}H-puromycin is taken to be analogous to normal peptide bond formation (Yarmolinsky and de la Haba, 1959; Morris and Schweet, 1961; Allen and Zamecnik, 1962; Nathans, 1964; Traut and Monro, 1964; Gottesman, 1967) and any enzymatic reaction which makes more polypeptides reactive with ^{3}H-puromycin is assumed to represent translocation. These data show that the T2 fraction contains translocase activity and agree with the findings of Skogerson and Moldave (1968)[3].

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[2]Abbreviations: T1 and T2, aminoacyl transferases I and II; MET, 2-mercaptoethanol; TCA, Trichloracetic acid.

[3]During the course of these studies we learned that Skogerson and Moldave were utilizing the same technique with similar findings. We thank them for making their manuscript available to us prior to its publication.

Diphtheria toxin, with NAD, reversibly inhibits the transferase activity of T2 (Collier and Pappenheimer, 1964; Collier, 1967; Goor and Pappenheimer, 1967a, 1967b; Goor, et al., 1967), apparently by catalyzing the transfer of the ADP-ribose moiety of NAD to T2 (Honjo, Nishizuka and Hayaishi, 1968). Diphtheria toxin, with NAD, also prevented the usual stimulation of the puromycin reaction by both crude soluble factors and partially purified T2.

MATERIALS AND METHODS

^{3}H-puromycin (^{3}H-methoxy, 1.11 C/mmole) and ^{14}C-L-phenylalanyl s RNA from E. coli (U. L., 177 mμC/mg) were obtained from New England Nuclear Corp. ^{14}C-L-leucyl sRNA (U. L., 42 mμC/mg) was purified from rat liver (Moldave, 1963). Crystalline ribonuclease was obtained from Worthington Biochemical Corp. and pronase from CalBiochem. Diphtheria toxin was purified as described by Goor and Pappenheimer (1967a). T1 and T2 were purified from rat liver and resolved by the method of Gasior and Moldave (1965), and assayed by incorporation of ^{14}C amino acids from aminoacyl-sRNA into protein. Rat liver ribosomes were prepared by a slight modification of the method of Wettstein, et al. (1963). The ribosomal pellets were then either washed five times with 0.5 M NH_4Cl, 0.01 M $MgCl_2$ and 0.05 M Tris, pH 7.3 at 25°, or centrifuged through a sucrose gradient containing 0.5 M NH_4Cl (Skogerson and Moldave, 1967). Protein was determined colorimetrically (Lowry, et al., 1951).

For the reaction with puromycin, ribosomes containing 0.05 to 0.1 mg protein were reacted with 1.8 μM ^{3}H-puromycin, 6 mM $MgCl_2$, 80 or 320 mM NH_4Cl, 50 mM Tris, pH 7.3 at 25°, in a final volume of 0.5 ml. In some reactions, 0.35 mM GTP and 30 mM MET were also present. The reaction was stopped with 5 ml of 5% TCA, 0.3 mg pH 5 supernatant was added as carrier protein, and the mixtures heated at 90° for 15 minutes. After centrifugation the supernatant was discarded and the precipitates collected on glass fiber filters (Whatman GF/C) and washed with 15 ml 5% TCA, 5 ml 95% ethanol, 5 ml ethanol-chloroform-ether (2:1:2) and 5 ml ether. The filters were transferred to vials and the precipitates dissolved with 0.2 ml NCS (Nuclear

Chicago Corp.), and counted in a toluene-based solution (Liquifluor, Pilot Chemicals, Inc.) in a Packard Tri-Carb model 314E scintillation counter with an efficiency of 30% for ^{3}H.

RESULTS AND DISCUSSION

The formation of peptidyl-^{3}H-puromycin required Mg^{++} and either NH_4^+ or K^+, and reached completion in ninety minutes at either 23° or 37°. The extent of the reaction was increased about 50% at 320 mM NH_4^+ (Skogerson and Moldave, 1968) or K^+, although overall protein synthesis is optimal at 80 mM. MET and GTP did not, by themselves, increase the amount of peptidyl-^{3}H-puromycin formed, but when combined with pH 5 supernatant a 50-70% increase in hot TCA insoluble radioactivity was observed (Schneider, et al., 1968). Assuming a molecular weight of 4×10^6 for rat liver monosomes (Wettstein, et al., 1963), 15-20% of the ribosomes contributed a TCA precipitable polypeptide to puromycin in the presence of pH 5 supernatant. Cycloheximide inhibited peptidyl-^{3}H-puromycin formation both with and without pH 5 supernatant. When T1 and T2 were completely resolved (Fig. 1), the fraction con-

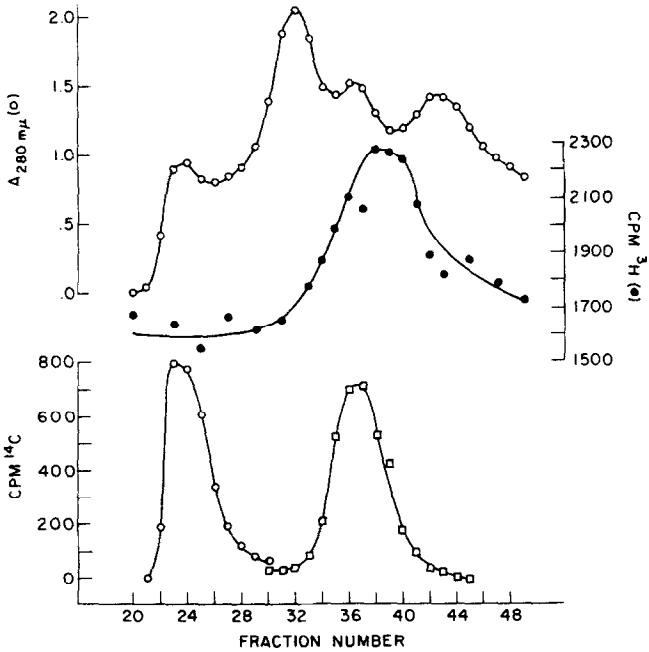

Fig. 1. Coincidence of T2 and the stimulatory activity for the puromycin reaction. T1 and T2 were separated on Sephadex G-200 (superfine) by upward flow elution with buffer containing 0.05 M Tris pH 7.3, 0.15 M KCl, 10^{-3} M dithiothreotol and 10^{-4} M EDTA. 100 mg of partially purified transfer factors (Gasior and Moldave, 1965) were applied to a column 2.5 cm x 31 cm which had been equilibrated with the above buffer, and 2.5 ml fractions were collected at a flow rate of 2.5 ml/hr. T1 (-○-, lower curve) was assayed with an excess of T2 and T2 (-□-, lower curve) was assayed with an excess of T1 as described in Methods. Peptidyl-^{3}H-puromycin formation (-●-) was assayed in the presence of 0.025 ml of each fraction with ribosomes containing 0.05 mg protein, 320 mM NH_4^+ and other components as described in Methods. Incubations were done in duplicate for 90 minutes at 37°.

taining T2 was the one which stimulated the puromycin reaction.

Allen and Zamecnik (1962) found that concentrations of ^{12}C-puromycin as high as 600 μM released only 20-30% of ^{14}C-labeled peptide chains from rabbit reticulocyte ribosomes and if ^{14}C-puromycin was incubated with unlabeled ribosomes, two-thirds of the ^{14}C remained bound to the ribosomes after centrifugation at 105,000 g for 60 minutes. In the experiments reported in this study the total hot TCA precipitable radioactivity was determined without separating the ribosomal and supernatant material. When the ribosomes were removed by centrifugation at 105,000 g for 120 minutes, 2/3 of the hot TCA insoluble radioactivity was recovered with the ribosomal pellet whether or not soluble factors were present. The recovery of hot TCA insoluble radioactivity in the ribosomal fraction was not altered by treatment with ribonuclease, but was almost completely eliminated following treatment with

TABLE I

Effect of T2, Diphtheria Toxin and NAD on Formation of Peptidyl-^{3}H-Puromycin with Rat Liver Ribosomes

Reaction System	Hot TCA insoluble ^{3}H		Increase over complete system	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
With GTP and MET	CPM		%	
Complete	2076	2410	0	0
-ribosomes	44	-	-	-
+T2	3226	3572	55	48
+T2+Toxin	3451	-	66	-
+T2+NAD	3029	-	46	-
+T2+Toxin+NAD	2416	2524	16	4.7
+Toxin	2698	2788	30	16
+NAD	2050	2605	0	8.1
+Toxin+NAD	2723	2739	31	14
Without GTP and MET				
Complete	-	2504	-	0
+T2	-	2580	-	3.0
+Toxin	-	2968	-	19
+Toxin+NAD	-	3080	-	23

The complete 0.5 ml reaction mixture included ribosomes containing 0.074 mg protein, 320 mM NH_4Cl and other components as described in Methods. In some reactions 0.4 mg pooled T2, 0.13 mg (50 Lf units) of purified diphtheria toxin or 0.09 mM NAD were added as shown. Zero time values which were obtained by adding TCA before adding the ribosomes averaged 50 CPM and were not altered by the presence of T2 or Toxin. Incubations were performed in duplicate for 90 minutes at 37°.

pronase. Thus, the hot TCA insoluble ^{3}H bound to the ribosomes is most likely peptidyl-^{3}H-puromycin. It is not understood why some peptidyl-^{3}H-puromycin remains bound to the ribosomes.

The experiments in Table I demonstrate that neither diphtheria toxin nor NAD alone significantly inhibited the increase in peptidyl-^{3}H-puromycin formation caused by T2. When added together, however, this stimulation was largely eliminated. One unexpected finding was the apparent stimulation of peptidyl-^{3}H-puromycin formation by diphtheria toxin in the absence of T2. Unlike the stimulation by T2, this did not require GTP or MET (Table I). We are unable to interpret this result at present.

The fact that both aminoacyl transferase and translocase activities reside in the T2 fraction, and that both activities are inhibited by diphtheria toxin, with NAD, suggests that these activities require a common protein.

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