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EFFECT OF DETERGENT ON PUROMYCIN-MEDIATED RELEASE OF NASCENT PEPTIDES FROM MAMMALIAN LIVER RIBOSOMES

Nuran Nabi*, Toshiaki Oda, and Tsuneo Omura

Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, Japan

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Summary: Puromycin-mediated *in vitro* release of nascent peptides from free and bound ribosomes prepared from rat livers was markedly enhanced by the presence of a non-ionic detergent, Triton X-100, in the incubation medium, and the enhancement by the detergent was more pronounced for bound ribosomes than for free ribosomes. Same results were obtained when nascent peptides were labeled either *in vivo* by radioactive amino acids or *in vitro* by radioactive puromycin.

Nascent peptides can be released from ribosomes *in vitro* by the reaction with the aminoacyl tRNA analogue puromycin (1-3). This reaction has been widely used in studying the subunits of polyribosomes (4), ribosome-membrane interaction (5,6), the sidedness of discharge of nascent peptides from membrane-bound ribosomes (7-10), glycosylation of nascent peptides (11), and the site of synthesis of specific enzymes (12,13). Proper interpretation of the results of those investigations depends, however, on the efficiency of the puromycin-mediated release of nascent peptides from ribosomes. Hence, optimization of the release of nascent peptides by puromycin is an essential prerequisite to the usefulness of this method.

In eukaryotic cells, two types of ribosomes, free and membrane-bound, are functional in protein synthesis. Since these two populations of ribosomes synthesize different kinds of proteins (14-19), including hydrophilic and hydrophobic ones, the efficiency of puromycin-mediated release of nascent peptides may vary between them. It seems possible that some portion of puromycin-released peptides remain attached to ribosomes through their hydrophobic interaction. We studied the effect of detergents on the

*on leave from the Department of Biochemistry, University of Dacca, Bangladesh

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release of nascent peptides by puromycin from free and bound ribosomes prepared from rat liver, and we present here the evidence that the puromycin-mediated release of nascent peptides from the ribosomes was significantly enhanced by a non-ionic detergent, Triton X-100, and that the enhancement was more pronounced for bound ribosomes than for free ribosomes.

MATERIALS AND METHODS

Male rats of Sprague-Dawley strain were fed with a commercial rat chow and used at a body weight of 180-230 g. DL-(1-^{14}C)-leucine was purchased from Daiichi Chemicals Co., Tokyo. L-(U-^{14}C)-glutamic acid and (8-^{3}H)-puromycin were obtained from the Radiochemical Centre, Amersham. Ribonuclease inhibitor was prepared from rat liver according to the method of Gribnau et al. (20). All other reagents used were of reagent quality.

For labeling nascent peptides in vivo, rats were starved for 18 hr, and ^{14}C-leucine (20 μCi per 100 g body weight) or ^{14}C-glutamic acid (5 μCi per 100 g body weight) in 0.9 % NaCl was injected to the animals through the tail vein. Two minutes later, the animals were killed by decapitation to prepare free and bound ribosomal fractions from their livers as follows. The livers were homogenized in 5 volumes of 0.88 M sucrose containing TMK buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 25 mM KCl). The homogenate was centrifuged at 15,000 x g for 20 minutes to prepare the post lysosomal supernatant. 18 ml of the supernatant was layered over a discontinuous gradient of sucrose consisting of 7 ml of 1.3 M sucrose in TMK buffer and 8 ml of 2.1 M sucrose in TMK buffer. Each sucrose layer also contained 10 units/ml of ribonuclease inhibitor and 100 μg/ml of heparin. The gradient was then spun at 174,000 x g for 4 hr. Rough microsomes formed a band at the interface between 1.3 M and 2.1 M sucrose layers, while free ribosomes formed a pellet at the bottom of the tube. The band of rough microsomes was carefully collected and diluted two fold with TMK buffer containing ribonuclease inhibitor and heparin. Triton X-100 was then added to the suspension of rough microsomes to give a final concentration of 1 %. 25 ml portion of the detergent-treated rough microsomes was layered over 9 ml of 1.5 M sucrose containing TMK buffer, ribonuclease inhibitor, and heparin. The tube was spun at 174,000 x g for 2.5 hr. The bound ribosomes formed a pellet at the bottom. The supernatant was discarded, and the wall of the tube was wiped several times. The surface of ribosomal pellets (free and bound ribosomes) were rinsed with distilled water. Pellets of free and bound ribosomes were separately suspended in a small volume of TMK buffer.

The discharge of nascent peptides from ribosomes was carried out as follows. Freshly prepared free and bound ribosomes were used in all experiments. 2 ml of the suspensions of free ribosomes or bound ribosomes (50-70 absorbancy at 260 nm) were incubated at 0° C with puromycin. The reaction mixture contained 50 mM Tris-HCl (pH 7.6), 2.5 mM $MgCl_2$, 0-1000 mM KCl as indicated, and 100 μM puromycin. After 30 minutes incubation, Triton X-100 was added to the reaction mixture to give final concentrations of 0-0.5 % as indicated. The incubation mixture was then centrifuged at 100,000 x g for 2 hr to sediment ribosomes, and a portion of the supernatant was counted in toluene-Triton X-100 scintillant to measure radioactivity.

For labeling nascent peptides in vitro with ^{3}H-puromycin, free and bound ribosomes were prepared from rat livers as described above without the injection of radioactive amino acids to the animal. The discharge of nascent peptides from ribosomal fractions was carried out as described above except that the concentrations of KCl and puromycin in the incubation mixture were 500 mM and 20 μM, respectively, and that 2 μCi of radioactive puromycin was included in each 2 ml of the mixture.

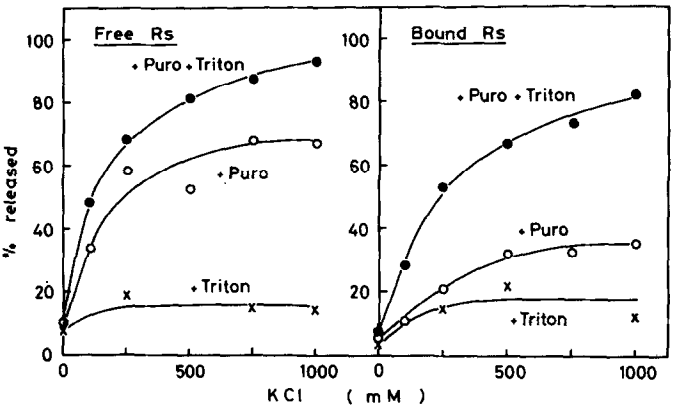

Fig. 1. Effect of Triton X-100 on Puromycin-mediated Release of ^{14}C-Leucine-labeled Nascent Peptides from Free Ribosomes (left) and Bound Ribosomes (right). Free and bound ribosomes (Rs) were separately incubated with puromycin in the presence of various concentrations of KCl, and the incubation mixtures were centrifuged at 100,000 x g with (●) or without (o) the addition of 0.5 % of Triton X-100 as described in the text. In control experiments (x), ribosomes were incubated with KCl and Triton X-100. The release of nascent peptides (radioactivity) into the supernatants is shown in the figure as the percentages of the total.

RESULTS AND DISCUSSION

The enhancing effect of Triton X-100 on puromycin-mediated release of nascent peptides from free and bound ribosomes is shown in Fig. 1. The release of ^{14}C-leucine-labeled nascent peptides increased from 65 % to 90 % when the incubation of free ribosomes with puromycin in the presence of 1000 mM KCl was followed by addition of Triton X-100 to a final concentration of 0.5 % (Fig. 1, left). On the other hand, the release of leucine-lebeled nascent peptides from bound ribosomes was enhanced from 35 % to 85 % under the identical condition (Fig. 1, right). Enhancement of puromycin-mediated release of nascent peptides from ribosomes by the detergent was also noticed when radioactive L-glutamic acid, a polar amino acid, was used as the label of the nascent peptides. Results shown in Fig. 2 confirm that Triton X-100 has same efficacy in the puromycin-mediated release of glutamic acid-labeled nascent peptides from bound ribosomes as when the nascent peptides were labeled with radioactive leucine, a non-polar amino acid (Fig. 1).

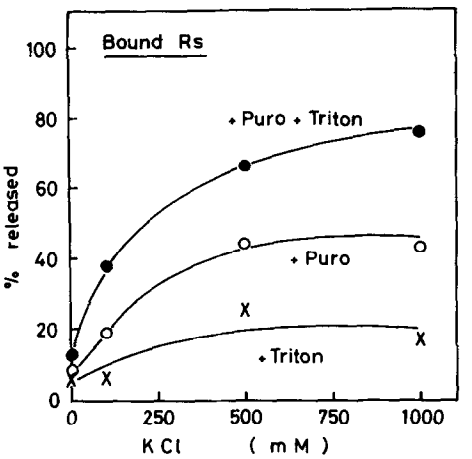

Fig. 2. Effect of Triton X-100 on Puromycin-mediated Release of ^{14}C-Glutamic Acid-labeled Nascent Peptides from Bound Ribosomes. Experimental procedures were the same as described in the legend to Fig. 1 except that radioactive glutamic acid was used in labeling nascent peptides in vivo. Symbols in the figure are the same as in Fig. 1.

Both in the presence and absence of Triton X-100, puromycin-mediated release of nascent peptides from ribosomes was dependent on the concentrations of KCl in the media. The results in the absence of the detergent (Figs. 1 and 2) are in agreement with an earlier report by Blobel and Sabatini (4). When ribosomes were incubated with either puromycin and Triton X-100 or KCl and Triton X-100, only about 15 % of radioactivity were released from ribosomes (Figs. 1 and 2). Triton X-100 enhanced the release of nascent peptides from ribosomes only when both puromycin and KCl were present in the incubation mixture. The minimum concentration of the detergent needed for the optimal release of nascent peptides was very low. As low as 0.05 % of the detergent was sufficient for the optimal enhancement (Fig. 3). Other non-ionic detergents such as Tween 20 were also equally effective in enhancing the puromycin-mediated release of nascent peptides from ribosomes. The effect of Triton X-100 was same when it was added to the incubation mixture before or after the incubation with puromycin.

The enhancing effect of Triton X-100 on the release of nascent peptides from ribosomes was also confirmed when the nascent peptides were labeled in

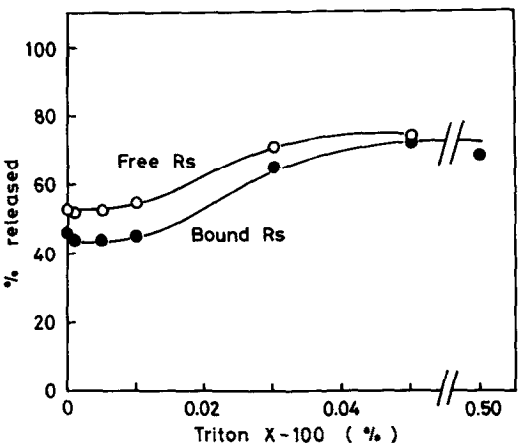

Fig. 3. Effect of Triton X-100 Concentrations on Enhancement of Puromycin-mediated Release of Nascent Peptides from Free and Bound Ribosomes. Free (o) and bound (•) ribosomes were incubated with puromycin in the presence of 500 mM KCl and various concentrations of Triton X-100. Other experimental conditions are described in the legend to Fig. 1.

vitro by incubating ribosomes with ^{3}H-labeled puromycin (Table I). In this experiment, the concentration of puromycin was 20 μM since it could be lowered to as low as 10 μM without significant decrease in the extent of release of nascent peptides under the condition employed. When ribosomes were treated stepwise firstly with puromycin only and then with Triton X-100, the sum of the nascent peptides fractions released at the first step (fraction S_1) and the second step (fraction S_2) was almost the same as when the ribosomes were treated with puromycin in the presence of Triton X-100 (fraction S_3). Therefore, the enhancing effect of Triton X-100 was not due to the promotion of the reaction of puromycin with ribosome-bound nascent peptides, but to the increased detachment of peptidyl puromycin from ribosomes.

We examined and compared the properties of the nascent peptides recovered in S_1 and S_2 fractions. The size distribution of the nascent peptides in these two fractions, as examined by SDS-polyacrylamide gel electrophoresis, were not much different from each other although S_2 showed

Table I.

Release of ^{3}H-Puromycin-labeled Nascent Peptides from Free and Bound Ribosomes.

	Radioactivity of released peptides		
fractions	S_1	S_2	S_3
	dpm x 10^{-3}		
Free ribosomes	66.5	40.0	126.0
(ratio)	(1.0)	(0.6)	(1.9)
Bound ribosomes	43.5	42.2	93.5
(ratio)	(1.0)	(1.0)	(2.1)

Free and bound ribosomes were separately treated with ^{3}H-labeled puromycin as described in the text. S_1 and S_2 fractions were obtained by treating ribosomes stepwise first with radioactive puromycin and then with Triton X-100. After each treatment, ribosomes were sedimented by centrifugation at 100,000 x g for 2 hr to obtain S_1 and S_2 fractions, respectively, in which released nascent peptides were recovered. S_3 fraction was prepared by incubating ribosomes with both radioactive puromycin and Triton X-100.

somewhat higher proportion of smaller nascent peptides than S_1 (data not shown). Hydrophobic column chromatography of S_1 and S_2 using alkyl Sepharose columns (21) also did not show a significant difference in hydrophobicity between them (data not shown). However, immunoprecipitation of specific nascent peptides (18) by antibodies against several liver proteins revealed a considerable concentration of certain kinds of nascent peptides in either one of S_1 and S_2. The contents of nascent serum albumin peptides in S_1 and S_2 obtained from bound ribosomes were 15 % and 4 %, respectively. The nascent peptides of NADPH-cytochrome c reductase, which is synthesized by both free and bound ribosomes (18), consists of 0.6 % of S_1 and 1.3 % of S_2, which were both prepared from free ribosomes. These results, which will be reported in detail elsewhere, indicate that the retension of peptidyl puromycin by ribosomes in the absence of detergents is significantly different from one species of nascent peptides to another. The use of a detergent seems to be indispensable to the use of puromycin-mediated in

vitro release of nascent peptides in studying the biosynthesis of specific enzyme proteins by ribosomes of mammalian tissues.

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