MECHANISM OF STIMULATION OF POLYPHENYLALANINE SYNTHESIS BY SPERMIDINE

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SUMMARY: It is shown that the stimulation of polyphenylalanine synthesis by spermidine is due mainly to the stimulation of initiation of polypeptide synthesis by following reasons: 1) the binding of poly(U) to ribosomes was stimulated more by spermidine than the binding of Phe-tRNA to ribosomes, and 2) the number of polyphenylalanine chains was increased more by spermidine than the extension of the chain length. In addition, it is shown that 30S ribosomal subunits are responsible for the stimulation of polyphenylalanine synthesis by spermidine.

Recently we have reported that polyamines are necessary for maximum polypeptide synthesis in E. coli and rat liver cell-free systems and that the increase of polyphenylalanine synthesis by SPD occurs at the level of Phe-tRNA binding to ribosomes and not at the level of peptide bond formation and translocation (1). It has been reported also that SPD is a necessary cofactor of polyphenylalanine synthesis in B. thur. (2). Therefore, the effect of SPD on the Phe-tRNA binding to ribosomes has been studied in detail using E. coli and B. thur. ribosomes.

MATERIALS AND METHODS

Materials - Dialyzed ribosomes from E. coli B and B. thur. Berliner, Sephadex G-50 treated E. coli S-100 (S-S100), and [^{14}C] Phe-tRNA were prepared as described previously (1,3,4). The preparation of ribosomal subunits (30S and 50S) was carried out according to the procedure of Takeda et al. (5). Partially purified EF-G was prepared according to Lucas-Lenard and Lipmann (6).

Procedures for polyphenylalanine synthesis and non-enzymatic binding of [^{14}C]Phe-tRNA to E. coli 70S ribosomes or 30S subunits - Polyphenylalanine synthesis was carried out as described previously (1). Non-enzymatic binding of Phe-tRNA to 70S ribosomes or 30S

Abbreviations: SPD, spermidine; B. thur., Bacillus thuringiensis

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subunits was carried out as described previously (1) except that the reaction mixture contained 100 mM NH_4Cl and 2 A_{260} units of 70S ribosomes or 30S subunits. When poly(U) independent binding of [^{14}C]Phe-tRNA to ribosomes was measured, the reaction mixture (0.2 ml) contained 5 A_{260} units of ribosomes, and poly(U) was omitted from the reaction mixture.

Procedure for determining [^{3}H]poly(U) binding to E. coli ribosomes - The reaction mixture (0.5 ml), containing 80 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 100 mM NH_4Cl, 3.75 A_{260} units of E. coli ribosomes, 50 µg of [^{3}H]poly(U) (100,000 cpm, Schwarz/Mann), and Mg^{2+} and SPD at the specified concentrations, was incubated at 30°C for 15 min. The complex of 70S ribosomes and [^{3}H]poly(U) was separated from unbound [^{3}H]poly(U) by sucrose density gradient centrifugation. A 0.45-ml aliquot of the reaction mixture was placed on top of a 5 to 20% sucrose gradient (4.5 ml) in 80 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, and Mg^{2+} and SPD at the specified concentrations. The tube was centrifuged in a Hitachi RPS-40 rotor for 2 hr at 39,000 rpm. After the centrifugation, ten-drop fractions were collected from the bottom of the tube. Ribosome-bound [^{3}H] poly(U) in each fraction was measured by the counting of the cold trichloroacetic acid insoluble radioactivity.

Procedure for the binding of [^{14}C]Phe-tRNA to the complex of poly(U) and E. coli ribosomes - The reaction mixture (0.5 ml), containing 80 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 100 mM NH_4Cl, 14 mM Mg^{2+}, 20 A_{260} units of E. coli ribosomes, and 300 µg of poly(U), was incubated at 30°C for 15 min. The complex of 70S ribosomes and poly(U) was separated by sucrose density gradient centrifugation as described above. The complex of poly(U) and ribosomes thus obtained was used for the [^{14}C]Phe-tRNA binding experiments. The reaction mixture (0.2 ml) contained 80 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 100 mM NH_4Cl, 1 A_{260} unit of the complex of poly(U) and ribosomes, 20,000 cpm of [^{14}C]Phe-tRNA, and Mg^{2+} and SPD at the specified concentrations. The bound [^{14}C]Phe-tRNA was measured by the procedure of Nirenberg and Leder (7).

Puromycin reactivity of the complex of [^{14}C]Phe-tRNA, poly(U) and ribosomes prepared in the presence or absence of SPD - The method used was essentially that of Igarashi et al. (8) except that the binding of [^{14}C]Phe-tRNA to ribosomes was carried out in the presence of Mg^{2+} and SPD at the specified concentrations. The reaction mixture (0.2 ml) for the formation of the puromycin derivatives of phenylalanine contained the following: 80 mM Tris-HCl (pH 7.5), 14 mM Mg^{2+}, 100 mM NH_4Cl, 1 mM dithiothreitol, 1.5 A_{260} units of the complex of [^{14}C]Phe-tRNA, poly(U) and ribosomes, and 1 mM puromycin. Where indicated, 20 µg of EF-G and 0.35 mM GTP were also added. After incubation at 30°C for 30 min, the puromycin derivatives formed were measured as described previously (8).

Determination of average chain length and number of polyphenylalanine chains synthesized in the presence or absence of SPD - The reaction mixture (1 ml) for polyphenylalanine synthesis was incubated at 30°C for 20 min or 60 min. After the incubation, 0.2 ml of 50 mM phenylalanine was added. This was followed by the addition of 10% trichloroacetic acid to a final concentration of 5%. The precipitate was treated with hot trichloroacetic acid and subsequently with dinitrofluorobenzene as described previously (9). The precipitate thus obtained was mixed with 0.7 ml of 6 N HCl and hydrolysis was carried out at 115 to 118° for 20 hr under a N_2 atmosphere. After hydrolysis, the mixture was diluted with 1.4 ml of water and dinitrophenyl (DNP) phenylalanine was extracted three times with 3 ml of ether. The ether phase was concentrated in

Table 1. Effect of spermidine on [^{3}H]poly(U) binding to E. coli ribosomes and poly(U) dependent or independent binding of [^{14}C]Phe-tRNA to E. coli ribosomes.

Ions (mM)		Bound [^{3}H] poly(U)	Bound [^{14}C]Phe-tRNA	
Mg^{2+}	SPD	$\left(\frac{\text{cpm}}{A_{260}\text{ unit of ribosomes}}\right)$	Binding to the complex of poly(U) and ribosomes $\left(\frac{\text{cpm}}{A_{260}\text{ unit of ribosomes}}\right)$	Poly(U) independent binding to ribosomes $\left(\frac{\text{cpm}}{5\ A_{260}\text{ units of ribosomes}}\right)$
14	-	934	1204	214
8	4	4585	1943	572

Procedures were as described in "Materials and Methods". Ions specified in the table were the optimal concentration for polyphenylalanine synthesis.

vacuo at room temperature, placed on a paper disc, and counted for radioactivity. The aqueous phase was neutralized with 0.3 ml of 10 N NaOH and a 0.2-ml aliquot was counted for radioactivity. The number of polyphenylalanine chains synthesized (p moles of NH_2 terminal-groups) was calculated from the radioactivity of the ether phase on the basis of 1 pmole equaling 429 cpm. Average chain length was calculated as total radioactivity of ether and aqueous phase divided by the radioactivity of the ether phase.

RESULTS

Effect of SPD on the binding of poly(U) or Phe-tRNA to ribosomes - As shown in Table 1, the SPD stimulation of poly(U) binding to ribosomes was about 4.9 fold, and the stimulation of Phe-tRNA binding to the complex of poly(U) and ribosomes was about 1.6 fold. The poly(U) independent binding of Phe-tRNA to ribosomes was also stimulated by SPD (Table 1). These results suggest that the binding of mRNA to ribosomes is influenced more by SPD than the binding of aminoacyl-tRNA to ribosomes. Next, an inves-

Table 2. Effect of spermidine on the binding of [^{14}C]Phe-tRNA to *E. coli* 30S ribosomal subunits and 70S ribosomes.

Ribosomal components	Incubation time (min)	Ions(mM) Mg^{2+}	Ions(mM) SPD	Bound [^{14}C] Phe-tRNA (cpm)	% Stimulation by SPD
Ribosomes	5	14	-	2351	
		8	4	4352	185
	10	14	-	3079	
		8	4	5296	172
30S Subunits	5	14	-	5702	
		8	4	11330	199
	10	14	-	7053	
		8	4	13405	190

Procedure was as described in "Materials and Methods". Ions specified in the table were the optimal concentration for polyphenylalanine synthesis.

tigation was made to determine whether the stimulation by SPD of aminoacyl-tRNA binding to P site is the same as that to A site. It has been suggested that there are two ribosomal sites for the binding of aminoacyl-tRNA (P and A sites) on 70S ribosomes (9-13), while there is only one aminoacyl-tRNA binding site on 30S ribosomal subunits (P site) (14-17). Therefore, the degree of stimulation by SPD on the binding of Phe-tRNA to 70S ribosomes and to 30S ribosomal subunits was compared. As shown in Table 2, the stimulation by SPD of Phe-tRNA binding to 30S ribosomal subunits was somewhat greater than the stimulation of binding to 70S ribosomes. If one assume that SPD stimulates the binding of Phe-tRNA to P site slightly more than the binding of Phe-tRNA to A site, the stimulation by SPD of the formation of a puromycin derivative in the absence of EF-G and GTP should be slightly greater than the stimulation of binding of Phe-tRNA to ribosomes. As shown

Table 3. Puromycin reactivity of the complex of [^{14}C]Phe-tRNA, poly(U) and ribosomes prepared in the presence or absence of spermidine.

Ions (mM)		[^{14}C]Phe-tRNA Bound (cpm)	Puromycin derivative formed (cpm)	
Mg^{2+}	SPD		No addition	+GTP and EFG
14	-	1403	256	758
8	4	2523	510	1439

Procedures were as described in "Materials and Methods". Ions specified in the table were the optimal concentration for polyphenylalanine synthesis.

in Table 3, the stimulation by SPD of the binding of Phe-tRNA to ribosomes was about 1.8 fold, while the stimulation of the formation of puromycin derivative was about 2.0 fold.

Average chain length and number of polyphenylalanine chains synthesized in the presence of Mg^{2+} or of Mg^{2+} plus SPD - When the reaction mixture was incubated for 20 min, the stimulation by SPD of number of polyphenylalanine chains (P moles of NH_2-terminal groups) synthesized with E. coli or B. thur. ribosomes was 1.70 and 3.89 fold, respectively, while the SPD stimulation of average chain length of polyphenylalanine synthesized with E. coli or B. thur. ribosomes was 1.29 and 1.41 fold, respectively (Table 4). This suggests that the initiation step of polyphenylalanine synthesis is influenced more by SPD than is chain elongation.

Ribosomal subunits responsible for stimulation of polyphenylalanine synthesis by SPD - Polyphenylalanine synthesis was carried out by reconstituted systems in which the two kinds of ribosomal

Table 4. Average chain length and number of polyphenylalanine chains synthesized in the presence of Mg^{2+} or Mg^{2+} plus spermidine.

Ribosomes	Time (min)	Ions(mM)		Radioactivity (cpm)		p moles of NH_2-terminal groups	Average chain length
		Mg^{2+}	SPD	aqueous phase	ether phase		
E. coli	20	14	-	34510	1673	3.90	21.6
		8	4	76412	2840	6.62	27.9
	60	14	-	75892	3123	7.30	25.3
		8	4	190281	4934	11.50	39.6
B. thur.	20	19	-	9466	692	1.61	14.7
		8	8	51132	2685	6.26	20.0
	60	19	-	16056	929	2.17	18.3
		8	8	88252	2755	6.42	33.0

Procedure was as described in "Materials and Methods". Ions specified in the table were the optimal concentration for polyphenylalanine synthesis.

subunits were of different bacterial origin. As shown in Table 5, polyphenylalanine synthesis was stimulated 5 fold by SPD with B. thur. 30S and E. coli 50S subunits, while the polyphenylalanine synthesis was stimulated 2 fold by SPD with E. coli 30S and B. thur. 50S subunits. This clearly suggests that 30S ribosomal subunits are responsible for the stimulation of polyphenylalanine synthesis by SPD.

DISCUSSION

The data presented show that the stimulation of polyphenylalanine synthesis by SPD is due mainly to the stimulation of initiation of polyphenylalanine synthesis. The binding of poly(U) to ribosomes was stimulated more by SPD than the binding of Phe-tRNA to ribosomes. The binding of Phe-tRNA to P site of ribosomes was stimulated slightly more by SPD than the binding to A site.

Table 5. Polyphenylalanine synthesis catalyzed by reconstituted systems containing ribosomal subunits from E. coli and B. thuringiensis.

Source of ribosomal subunits		Ions (mM)		[^{14}C]Phe incorporated	% Stimulation by
30S	50S	Mg^{2+}	SPD	(cpm)	SPD
E	E	13	-	5619	
		7	4	11444	204
E	B	18	-	7598	
		10	4	14639	193
		8	6	14096	186
B	E	21	-	690	
		9	6	3254	472
		6	8	3497	508
B	B	22	-	1027	
		7	8	5195	506

Polyphenylalanine synthesis was carried out under standard conditions, except that 0.3 A_{260} unit of E. coli (E) 30S or 0.75 A_{260} unit of B. thur. (B) 30S, and 0.6 A_{260} unit of E. coli or B. thur. 50S ribosomal subunits were added to the reaction mixture as specified in the table instead of 70S ribosomes. A 2.5 fold of B. thur. 30S ribosomal subunits was added to the reaction mixture because of the instability of these 30S subunits (22).

Furthermore, the number of polyphenylalanine chains synthesized was increased more than the extension of average chain length by the addition of SPD. Stimulation at the initiation level by polyamines has been reported by other workers who used reticulocyte or ascites ribosomes (18,19). However, the influence of SPD on the binding of Phe-tRNA to A site may gradually increase with incubation time, as judged by the increase of chain length of polyphenylalanine in the presence of SPD (Table 4). Atkins et al. (20) have reported that relatively high molecular weight polypeptides are made in the presence of polyamines when the reaction mixture is incubated for 2 hrs at 36°. This is in accordance with our results.

Since there are several steps involved in the formation of the initiation complex, it is of interest that the binding of mRNA to 30S ribosomal subunits is the step most influenced by SPD, and that SPD stimulation of polypeptide synthesis is dependent on the uracil content of mRNA (21). Experiments are now in progress to elucidate the effect of polyamines on the initiation of protein synthesis with natural mRNA.

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