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Spermidine Requirement for *Bacillus thuringiensis* Ribosomes in Cell-Free Phenylalanine Incorporation

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A cell-free system from *Bacillus thuringiensis* was found to actively incorporate phenylalanine into hot trichloroacetic acid-precipitable material in the presence of synthetic polynucleotide, ribosomes, S-100 supernatant, an energy-generating system, and guanosine triphosphate. Phenylalanine incorporation was absolutely dependent on the presence of spermidine in addition to magnesium ions, even when highly purified ribosomes were used. The spermidine effect could not be attributed to inhibition of nucleases. The ribosomal and supernatant fractions from *Escherichia coli* and *B. thuringiensis* could be substituted for each other in this system. The spermidine requirement was shown to be limited to the *B. thuringiensis* ribosome fraction.

A crystalline protein inclusion develops to maximum size over a several-hour period during the sporulation of *Bacillus thuringiensis* (13). Serological studies have shown that this crystalline protein (the parasporal body) is synthesized de novo (24) and that the onset of this synthesis corresponds closely with the initiation of sporulation (28). Interest in cell-free protein synthesis with extracts from this organism is enhanced because of the long time period over which the parasporal protein is produced in the cell. This communication describes the characteristics of a system in which extracts from *B. thuringiensis* could actively incorporate phenylalanine into hot trichloroacetic acid-insoluble polypeptide. These results reveal an unusual requirement of spermidine for *B. thuringiensis* ribosome function.

MATERIALS AND METHODS

Materials. Uniformly ^{14}C-labeled L-phenylalanine (355 μCi/μmole) was obtained from New England Nuclear Corp. Poly-U-^{3}H was purchased from Miles Laboratories. Adenosine triphosphate (ATP; disodium salt) and polyuridylic acid (poly-U) were products of Sigma Chemical Co. Phosphoenolpyruvate kinase (EC 2.7.1.40) was purchased from Boehringer & Soehne, Germany; spermidine from Aldrich Chemical Co., Inc.; phosphoenolpyruvate (tricyclohexylammonium salt) from General Biochemicals; ribonuclease (EC 2.7.7.16), amino acids, and bovine albumin from Nutritional Biochemical Company; guanosine triphosphate (GTP; disodium salt), and 2-mercaptoethanol from Mann Research Laboratories, Inc.; deoxyribonuclease (EC 3.1.4.5) from Worthington Biochemical Co.; putrescine and 2-hydroxy putrescine were kindly supplied by C. Hurwitz. Macaloid was a product of National Lead Co.

Organism and growth conditions. *Bacillus thuringiensis* var. *thuringiensis* Berliner was cultured aerobically at 30 C in a citrate-salts medium (41) containing 1.2 mM Mg^{2+} and enriched with trace metals, 0.1% glucose, and 0.033% casamino acids. Exponentially growing cells [0.19 to 0.28 mg (dry weight)/ml] were chilled rapidly and harvested by centrifugation (12,100 × *g* for 10 min, 0 C). The cells were washed twice with 0.01 M tris(hydroxymethyl)aminomethane-(Tris)-hydrochloride buffer (*p*H 7.6) containing 20 mM magnesium acetate, 50 mM ammonium chloride, 60 mM potassium chloride, and 6 mM 2-mercaptoethanol (Tris buffer A). Cells were used immediately to prepare cell-free extracts or were stored at −20 C for future use. *Escherichia coli* K-12 was grown in nutrient broth and the cells were prepared as for *B. thuringiensis*.

Preparation of cell-free extracts. The procedure for preparation of cell-free extracts was based on that developed by Nirenberg (26) for *E. coli*. All operations were carried out at 0 to 4 C. Washed cells were ground with twice their weight of alumina (A-305 from Aluminum Co. of America) in a prechilled porcelain mortar. After approximately 10 min of grinding, Tris buffer A, corresponding to about twice the cell weight (v/w), was added, and the paste was suspended evenly by gently stirring. After removal of intact cells and debris by centrifugation (20,000 × *g* for 20 min), purified Macaloid paste was added at a concentration of 0.5 g per 10 ml to reduce nuclease activity (32). The supernatant fluid resulting from centrifuging at

[1] This work represents part of a doctoral dissertation to be submitted by Li-ming Changchien.

20,000 × g for 10 min was finally centrifuged at 30,000 × g for 30 min. The upper four-fifths volume of this supernatant (S-30) was removed, and portions were quickly frozen in an acetone-dry ice bath and stored at −20 C until needed.

Preparation of ribosomes and S-100 supernatant. Ribosomes were prepared from S-30 extracts by centrifuging at 105,000 × g for 2 hr. The supernatant was removed by aspiration; pelleted ribosomes were washed twice with Tris buffer A and resuspended finally in Tris buffer A. *S*-100 supernatant fluid and ribosomes were stored in small fractions at −20 C.

Protein concentration was estimated by the method of Lowry et al. (20), with bovine serum albumin as a standard.

Conditions for amino acid incorporation. The exact composition of the complete reaction mixture is described in each table and figure. The assay was initiated usually by addition of 0.1 ml of S-30 extract before incubating at 37 C for 30 min in a total volume of 0.5 ml. The reaction was terminated by the addition of 4 ml of cold 10% trichloroacetic acid and 400 μg of bovine albumin as a carrier. After cooling at 0 C for 1 hr, the precipitated material was collected by centrifugation at 17,300 × g for 10 min. The pellet was washed once with cold 5% trichloroacetic acid, suspended in 4 ml of cold 5% trichloroacetic acid, heated at 90 C for 15 min (33), and then chilled in an ice bath for 30 min. The precipitate was collected on a membrane filter (HA, 0.45 μm, Millipore Corp.), washed four times with 5 ml of 5% acid, transferred to a scintillation vial, and dried under an infrared lamp. Scintillation solution (10 ml) containing per liter 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]-benzene in toluene was added to the vial, and the radioactivity of the sample was determined in an Ansitron liquid scintillation spectrometer. All samples were assayed in duplicate or in triplicate

RESULTS

Stability of cell-free extracts. Cell-free extracts quick-frozen in an acetone-dry ice bath retained activity for amino acid incorporation for at least 2 weeks when stored at −20 C. The activity of cell-free extracts prepared from frozen cells stored whole for 3 days at −20 C did not differ much from activity obtained with extracts of fresh cells. The cell-free extracts from late-log-phase cells had only 52% activity of that from early log-phase cells when measured in the standard way. Although the significance of this observation was not investigated further, only early or mid-log-phase cells were used in these experiments. *B. subtilis* ribosomes from late-log cells have been reported to possess decreased activity (15).

Properties of the incorporating system. The incorporation of phenylalanine into hot trichloroacetic acid-insoluble material required the presence of both ribosomes and S-100 supernatant (Table 1). Omission of the energy-generating system resulted in 97% loss of activity. In the absence of GTP, the incorporation was decreased by 35%. Ribonuclease (10 μg/ml) completely inhibited incorporation; however, deoxyribonuclease (40 μg/ml) had no effect on incorporation. No significant incorporation was observed in the absence of poly-U. Incorporation of phenylalanine into acid-insoluble material paralleled the concentration of poly-U in the assay up to the saturating concentration of 140 μg/ml. A almost 40-fold stimulation was obtained in phenylalanine incorporation over the incorporation without poly-U. In the presence of poly-U at a concentration of 100 μg/ml, the incorporation of phenylalanine at 37 C was linear for 30 min and was complete within 45 min. A very sharp temperature optimum for maximal phenylalanine incorporation was observed at 37 C. Incorporation at 20 C was only 18% of that obtained at 37 C and no incorporation was obtained when the reaction was carried out at 45 C or above. Optimal *p*H for amino acid incorporation in the system was 7.3. Incorporation at either *p*H 7.1 or 8 was decreased by 20 and 27%, respectively.

TABLE 1. *Requirements for incorporation of ^{14}C-phenylalanine into trichloroacetic acid-insoluble polypeptide in the cell-free system from Bacillus thuringiensis*

Conditions	^{14}C-phenylalanine incorporated[a]
Complete[b]	10.82
Minus ATP, PEP, and PEP kinase	0.28
Minus ribosomes	0.59
Minus supernatant	0.59
Minus poly-U	0.05
Minus GTP	7.02

[a] Values expressed as picomoles per milligram of total protein.

[b] The complete reaction mixture (0.5 ml) contained the following per ml: 10 μmoles of Tris-hydrochloride (*p*H 7.6), 60 μmoles of KCl, 50 μmoles of NH_4Cl, 20 μmoles of magnesium acetate, 5 μmoles of spermidine, 6 μmoles of 2-mercaptoethanol, 2.5 μmoles of adenosine triphosphate (ATP), 0.25 μmoles of guanosine triphosphate (GTP), 5 μmoles of phosphoenolpyruvate (PEP), 40 μg of pyruvate kinase, 100 μg of poly-U, 0.05 μCi (141 pmoles) of ^{14}C-phenylalanine, 0.05 μmoles each of the 19 other amino acids, and 0.2 ml of S-30 fraction (2.1 mg of protein). The reaction mixture was incubated at 37 C for 30 min and treated as described in Materials and Methods. The zero-time values, usually less than 0.3 pmoles/mg of protein, were subtracted.

Effect of monovalent cations on the poly-U-directed phenylalanine incorporation. In the absence of ammonium ions, the system required

quite high concentration of K^+ for maximum incorporation (0.12 M). Inclusion of ammonium ions in the system not only reduced the potassium concentration required for maximum incorporation, but also slightly increased the total incorporation compared to that without the addition of ammonium ions. An optimal 31.2 mM K^+ was observed for phenylalanine incorporation in the presence of 50 mM ammonium ions. If the potassium concentration was fixed at 31.2 mM, the system showed a broad optimum range of ammonium concentration with a maximum around 42 mM. The combination used in subsequent experiments was 31.2 mM K^+ plus 42 mM NH_4^+.

Absolute requirement for spermidine and magnesium in the incorporating system. The omission of spermidine from the system abolished phenylalanine incorporation almost completely (Fig. 1). Maximal incorporation of phenylalanine was obtained at a final spermidine concentration of 3 mM. The effect of spermidine could not be balanced by replacement with added Mg^{2+}. Very little incorporation of phenylalanine into trichloroacetic acid-insoluble material occurred when the Mg^{2+} concentration was below 4 mM. Concentrations higher than the optimal 13.6 mM were inhibitory. The absolute requirement for both Mg^{2+} and spermidine for the poly-U-directed incorporation of phenylalanine into hot acid-precipitable material is illustrated in Table 2. In the absence of spermidine, very little incorporation was observed, even though Mg^{2+} concentration in the system was increased up to 29.6 mM. On the other hand, no activity was found when Mg^{2+} was completely substituted by spermidine.

Stimulation on the incorporation of phenylalanine into hot trichloroacetic acid-precipitable material by spermidine in cell-free systems has been reported by several investigators (2, 4, 7, 15, 18, 22, 40). Martin and Ames (22) and Takeda (40) reported that spermidine caused a shift in the optimal concentration of Mg^{2+} for cell-free amino acid incorporation. Takeda (40) suggested that replacement of Mg^{2+} by polyamines could occur in *E. coli* during protein synthesis in vivo. In contrast, the cell-free system from *B. thuringiensis* was inactive in phenylalanine incorporation without the addition of spermidine and the addition of various concentrations of spermidine to the system did not alter the optimal concentration of 13.6 mM Mg^{2+} (Table 2). The spermidine requirement was shown to be only for the *B. thuringiensis* ribosome fraction by using a heterologous system with *E. coli* fractions (Table 3). Addition of spermidine resulted only in a modest stimulation of incorporation with the *E. coli* ribosomes and *B. thuringiensis* supernatant fraction. In general, phenylalanine incorporation was considerably increased when the *E. coli* supernatant fraction was substituted.

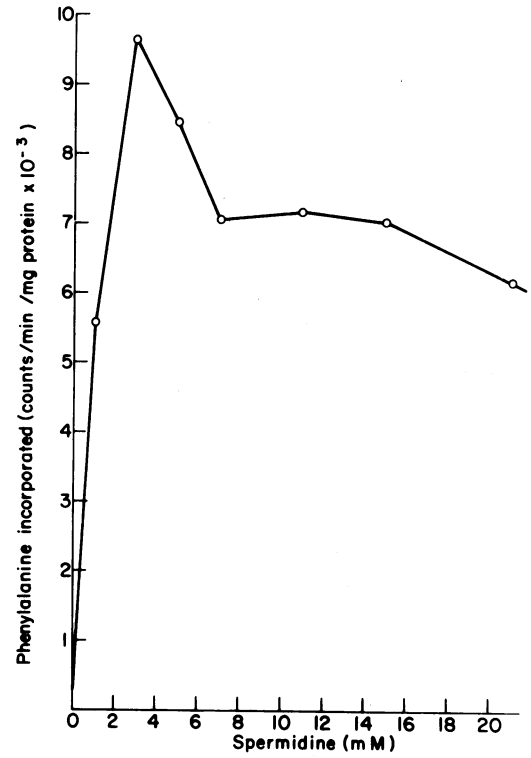

FIG. 1. *Effect of spermidine on the incorporation of ^{14}C-L-phenylalanine into trichloroacetic acid insoluble polypeptide in the cell-free system from B. thuringiensis. The composition of reaction mixture was as described for Table 1 except for the various spermidine concentrations noted above. The reaction mixture was incubated at 37 C for 30 min and treated as described in the text. Control value without poly-U was subtracted. The equivalent of 5,000 counts/min of ^{14}C-phenylalanine incorporated is 8.8 pmoles.*

The comparative stimulatory effects of spermidine, putrescine, and 2-hydroxyputrescine are shown in Table 4. Stimulation by either putrescine or 2-hydroxyputrescine was observed only at much higher concentrations than the optimal spermidine concentration (3 mM). At high concentrations, putrescine and 2-hydroxyputrescine gave equivalent stimulation, but considerably less than that obtained with spermidine. In the presence of optimal spermidine concentration, 2-hydroxyputrescine at high concentration (16 mM) inhibited phenylalanine incorporation by 25%. 2-Hydroxyputrescine has recently been identified as the polyamine associated with ribosomes of a pseudomonad (30).

Effect of spermidine on nuclease activity of

TABLE 2. *Requirement of magnesium and spermidine for the incorporation of ^{14}C-phenylalanine into trichloroacetic acid-insoluble polypeptide in the cell-free system from Bacillus thuringiensis*

Spermidine	^{14}C-phenylalanine incorporated[a] (pmoles/mg of protein) at magnesium concn (mM)								
	0.0	4.0	7.2	10.4	13.6	16.8	20.0	26.4	29.6
mM									
0.0			0.10	0.31	0.54	0.58	0.60	0.57	0.02
0.2					0.61				
0.5					3.36				
1.0					10.10				
2.0	0.02	0.61	3.62	11.14	13.41	9.94	7.50		
3.0	0.07	0.35	10.49	18.40	19.98	12.13	8.37		
4.0	0.00	0.95	6.18	10.07	15.82	11.06			
5.0		0.16	3.65	12.74	15.66	9.47			
7.0		1.69	9.00	12.07	14.82	10.79			
14.0	0.01								
20.0	0.00								

[a] The reaction mixture (0.5 ml) contained the following per ml: 31.2 μmoles of KCl, 42 μmoles of NH_4Cl, and the indicated concentrations of both Mg^{2+} ions and spermidine. Other components were as described for Table 1. The reaction mixture was incubated at 37 C for 30 min and treated as described in the text. Control values without poly-U were subtracted.

extracts. Spermidine has been reported to inhibit ribonuclease action and to stabilize ribosomes against autodegradation (8). Preparations of MS-2 viral ribonucleic acid (RNA) which could program our *E. coli* cell-free system yielded essentially no incorporation of phenylalanine with our *B. thuringiensis* system, possibly because of rapid nucleolytic breakdown by the *B. thuringiensis* extracts. However, the degradation of tritiated poly-U (35) was not inhibited by the level of spermidine utilized in our poly-U system (Table 5). Gel electrophoresis, by the procedure of Wilson (42), revealed nine ribonuclease bands in the S-100 fraction and eight ribonuclease bands in ribosome washings (31). Although nuclease activity is abundant, it cannot account for the spermidine requirement in the poly-U-directed system.

DISCUSSION

The requirements of the cell-free system from *B. thuringiensis* for phenylalanine incorporation into hot trichloroacetic acid-precipitable material are similar to those from *E. coli* (27), yeast (4), *Bacillus* species (2, 12, 15, 36), and *Staphylococcus aureus* (21) with respect to ribosomes, supernatant, and energy-generating system. Our results showed that the ribosomal and supernatant fractions from *E. coli* were qualitatively interchangeable with corresponding fractions from *B. thuringiensis*, despite the essentiallity of spermidine for the *B. thuringiensis* ribosomes.

The kinetic studies showed that the rate of incorporation at 37 C was essentially linear for 30 min and then approached completion within 45 min. The pattern of kinetic curve is very similar to those found for systems from other species of *Bacillus* (1, 36, 39). The reduced rate of incorporation may be due to breakdown of the poly-U by ribonuclease, a process which we showed to be unaffected by spermidine. Current studies in our laboratory have revealed that MS-2 viral RNA cannot program either the *B. thuringiensis* system or the *E. coli* system in the presence of *B. thuringiensis* ribosomes. Ribosome-bound nuclease activity has been found, which, although not similar to the *E. coli* ribonuclease V (19), presumably inactivates the initiator end of the natural messenger RNA. *Bacillus* species seem to have high nuclease activity which may account for the defective 30*S* ribosome component in *B. subtilis* (39) and the poor capability of *B. megaterium* spore extracts to incorporate amino acids (7). The presence of spermidine is reported to inhibit nuclease activity and therefore stimulate incorporation (7, 8, 15, 18), although the evidence presented in this paper indicates some other explanation is needed for the *B. thuringiensis* system.

Polyamines occur in many organisms (6, 37, 38) and extracted ribosomes have also been reported to contain polyamines (5, 17, 23, 29, 34). Many workers have reported that polyamines not only preserve ribosomal structural integrity, but also bring about the association of ribosomal subunits to 70*S* and 100*S* ribosomal particles (5, 6, 22, 29). Although polyamine stimulation of amino acid incorporation in cell-free systems is well documented (2, 4, 7, 10, 14, 15, 18, 22, 25, 40), no absolute requirement for spermidine has been

TABLE 3. *Effect of spermidine on the incorporation of ^{14}C-phenylalanine into trichloroacetic acid-insoluble material in the heterologous cell-free system from Bacillus thuringiensis and Escherichia coli*

Expt[a]	Spermidine	Supernatant fraction	Ribosomal fraction	^{14}C-phenylalanine incorporated[b]
	mM			
1	0	*E. coli* K-12	*B. thuringiensis*	0.11
	3	*E. coli* K-12	*B. thuringiensis*	8.40
2	0	*B. thuringiensis*	*B. thuringiensis*	0.02
	3	*B. thuringiensis*	*B. thuringiensis*	5.10
3	0	*B. thuringiensis*	*E. coli* K-12	2.55
	3	*B. thuringiensis*	*E. coli* K-12	4.40

[a] The reaction mixture (0.25 ml) contained the following per ml: 31.2 μmoles of KCl, 42 μmoles of NH_4Cl, 13.6 μmoles of magnesium acetate, 0 or 3 μmoles of spermidine, 0.1 ml of ribosomal fraction, and 0.1 ml of S-100 supernatant fraction. Other components were as described for Table 1. The ribosomal fraction from *B. thuringiensis*, purified by the method of Salas et al. (31), contained 0.08 mg of protein; the supernatant fraction from *B. thuringiensis* used in experiments 1 and 2 contained 0.5 mg of protein and that in experiment 3 contained 0.85 mg of protein; that from *E. coli* contained 0.4 mg of protein. The ribosomal fraction from *E. coli* was not purified and contained 0.52 mg of protein. The reaction mixtures were incubated at 37 C for 30 min and treated as described in the text. Control values without poly-U were subtracted.

[b] Values expressed as picomoles per milligram of protein.

reported previously for bacterial systems. In contrast, spermidine has been reported to inhibit incorporation in a *B. stearothermophilus* system and also to increase miscoding (9). The apparent function of spermidine in the *B. thuringiensis* system is clearly different from the function in other systems (14, 22, 40) in which spermidine appears to have a role very similar to that of Mg^{2+} in formation and preservation of the ribosomal integrity required for active amino acid incorporation in the cell-free system. Failure to observe either amino acid incorporation in the absence of spermidine or a shift in the optimal concentration of Mg^{2+} in the presence of spermidine suggests that spermidine may have a

TABLE 4. *Effect of different polyamines on the incorporation of ^{14}C-phenylalanine into trichloroacetic acid-insoluble polypeptide in the cell-free system from Bacillus thuringiensis*

Spermidine	Putrescine	2-(HO)-Putrescine	^{14}C-phenylalanine incorporated[a]
mM	mM	mM	
0	0	0	0.89
2	0	0	22.60
3	0	0	24.40
0	0	1	1.02
0	0	3	1.87
0	0	6	2.24
0	0	10	3.91
0	0	12	6.65
0	0	16	8.24
3	0	2	20.79
3	0	6	25.03
3	0	12	22.22
3	0	16	16.80
0	14	0	6.10
0	16	0	7.96

[a] The reaction mixture (0.5 ml) contained the following per ml: 13.6 μmoles of magnesium acetate, 31.2 μmoles of KCl, 42 μmoles of NH_4Cl, and indicated concentrations of spermidine, putrescine, and 2-hydroxyputrescine. Other components were as described for Table 1. Control values without poly-U were subtracted. Values expressed as picomoles per milligram of protein.

TABLE 5. *Effect of spermidine on ribonuclease activity in S-30 extracts from Bacillus thuringiensis*

Expt[a]	Spermidine	^{3}H-poly-U-hydrolyzed[b]
	mM	
1	0.0	3,331
	1.5	4,183
	3.0	3,913
2	0.0	3,505
	1.5	4,253
	3.0	4,091

[a] The procedure of Spahr (35) was used. Incubations were at 37 C for 20 min. The complete reaction mixture contained per ml: 10 μmoles of Tris-hydrochloride (*p*H 7.6), 31.2 μmoles of KCl, 42 μmoles of NH_2Cl, 13.6 μmoles of magnesium acetate, 6 μmoles of 2-mercaptoethanol, 0.1 ml of S-30 extracts containing 1.38 mg of protein, 12.5 nCi of ^{3}H-poly-U (specific activity, 78.1 μCi/mmole of *P*) and various concentrations of spermidine. Experiment 1 used extracts from late-log cells, whereas extracts from experiment 2 were from mid-log cells. Scintillation counting was in Bray's solution (3).

[b] Values expressed as counts per minute per milligram of protein.

specific role which cannot be fulfilled by magnesium. Preliminary experiments (C. Hurwitz, *unpublished data*) indicate that the *B. thuringiensis* S-100 extracts contain unbound 1 mM spermidine, which is a very high concentration. It seems reasonable that intracellular levels of spermidine might play an important role in controlling protein synthesis in vivo at the translation level in *B. thuringiensis*, as has been previously suggested for *E. coli* (16). In the presence of optimal concentration of spermidine in our *B. thuringiensis* system, 16 mM 2-hydroxyputrescine inhibited incorporation by 25%. This suggests that 2-hydroxyputrescine can compete with spermidine for sites in this cell-free polypeptide synthesis, but is much less effective. A *Pseudomonad* sp. has recently been shown to have 2-hydroxyputrescine, but no spermidine (30).

Endogenous amino acid incorporation in our system is quite low in the presence or absence of spermidine, but one might get some insight into in vivo spermidine function by studying its effect on incorporation of amino acids with isolated polysomes from *B. thuringiensis* (11).

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