*PROTEIN CHAIN INITIATION AND DEFORMYLATION IN B. SUBTILIS HOMOGENATES**

By Masao Takeda and Robert E. Webster

THE ROCKEFELLER UNIVERSITY

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Since formylmethionyl-tRNA (F-met-tRNA) was discovered by Marcker and Sanger,[1] much evidence has accumulated that F-met-tRNA participates in the initiation of protein synthesis in the cell-free system of *E. coli*.[2-6] In *B. subtilis* extracts, Horikoshi and Doi observed the formylation of met-tRNA and the incorporation of F-met into protein from F-met-tRNA.[7] They suggested, however, that an alanine derivative may be involved in the initiation of protein synthesis in *B. subtilis* because of the presence of alanine at the *N*-terminal end of 80 per cent of the bulk proteins of *B. subtilis*.[8]

To examine further and compare the mechanism of initiation and peptide bond formation in *B. subtilis* and *E. coli* cell-free systems, we chose as a model system AUG-directed formylmethionylpuromycin (F-met-puro) formation.[9]

Methods.—Ribosomes and supernatants: *B. subtilis* SB-19 (ATCC 15575) and *E. coli* B were grown and harvested as previously described.[10] *B. subtilis* cells (10 gm) were suspended in 0.01 *M* tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, 0.01 *M* Mg acetate containing 15% glycerol (50 ml), and were stored at dry-ice temperature. Ribosomes and S-150 were prepared as described,[10] with the exception that 10% glyc rol was present in the standard buffer (0.01 *M* Tris-HCl, pH 7.4, 0.01 *M* Mg acetate, and 0.0005 *M* dithiothreitol (DTT)). The ribosomal pellet was washed once and resuspended with the same buffer to the concentration 1000–1500 OD_{260} units/ml, and was stored in dry ice.

Protein was determined as previously described.[10]

Preparation of B. subtilis deformylase: *B. subtilis* ribosomes (2200 OD_{260} units) were suspended in 3 ml of 0.5 *M* NH_4Cl, 0.01 *M* Tris-HCl, pH 7.4, 0.001 *M* Mg acetate, and 0.0005 *M* DTT, and the mixture was shaken gently for 1 hr at 4°. After the ribosomes were removed by centrifugation at 150,000 × *g* for 3 hr, 2.3 vol of ammonium sulfate solution (saturated in 0.1 mM ethylenediaminetetraacetate (EDTA) at pH 7.0 at 0°) was added gradually to the supernatant. The precipitate was collected by centrifugation, dissolved in 0.01 *M* Tris-HCl, pH 7.4, 0.0005 *M* DTT, and dialyzed against 100 vol of the same buffer for 3 hr. This enzyme solution could be stored at −20° without loss of activity for at least 2 weeks.

Preparation of B. subtilis aminopeptidase: To 40 ml of *B. subtilis* S-150 was added 60 ml of saturated ammonium sulfate solution, and the precipitate was discarded after centrifugation. To the supernatant was added 100 ml of saturated ammonium sulfate solution; the precipitate was collected by centrifugation, then dissolved in 3 ml of 0.01 *M* Tris-HCl, pH 7.4, 0.0005 *M* DTT, and the resulting solution was dialyzed against 3 liters of the same buffer. Because EDTA inhibits the aminopeptidase, it should be omitted from the saturated ammonium sulfate solution. This enzyme preparation was stored at −20°.

Preparation of H^3-formyl-C^{14}-methionyl-tRNA: Stripped tRNA's from *B. subtilis* and *E. coli* were charged and formylated by using homologous S-150 extracts. The reaction mixture contained in a total volume of 2 ml: 0.075 *M* Tris-HCl, pH 7.4; 0.005 *M* NH_4Cl; 0.005 *M* DTT; 0.02 *M* Mg acetate; 0.004 *M* adenosine 5′-triphosphate (ATP); 0.005 *M* phosphoenolpyruvate (PEP); 0.1 mg protein of PEP-kinase; 20 mg (480 OD_{260} units) of *B. subtilis* tRNA or 10 mg (240 OD_{260} units) of *E. coli* tRNA; 2 mg protein of *B. subtilis* or *E. coli* S-150; 150 mμmoles of C^{14}-L-methionine (spec. act. 199 μc/μmole); and 13.7 mμmoles of H^3-formyltetrahydrofolic acid (THFA) (spec. act. 4.39 μc/μmole). The H^3-

formyl-THFA was prepared as described by Adams and Capecchi.[3] Each mixture was incubated for 30 min at 30°. F-met-tRNA was isolated as described,[10] except that the final dialysis of the F-met-tRNA solution was against 1000 vol of 0.001 *M* EDTA for 3 hr and then against 1000 vol of water overnight to remove the EDTA. The products had a specific activity of 0.545 mμmole of F-met per mg of *B. subtilis* tRNA, and 1.14 mμmoles of F-met per mg of *E. coli* tRNA. Contamination with C^{14}-methionyl-tRNA was 25% of the total C^{14}-methionine in both *B. subtilis* and *E. coli* preparations.

Binding of F-met-tRNA to ribosomes: ApUpG was prepared by the methods developed in the Ochoa laboratory.[11] Binding of F-met-tRNA to ApUpG-charged ribosomes was assayed by the Millipore filter technique described by Nirenberg and Leder.[12] The Millipore disks were counted with 10 ml of Bray's solution in a Nuclear-Chicago Mark 1 liquid scintillation counter.

Assay for F-met-puro and deformylation: F-met-puro and met-puro were measured by ethylacetate extraction according to Leder and Bursztyn.[9] Values represent the amount of either F-met-puro extracted at pH 5.5 by 1.5 ml of ethylacetate, or the sum of F-met-puro and met-puro extracted at pH 8.0 by 1.5 ml of ethylacetate.

Preparation of H^3-formyl-C^{14}-methionylpuromycin: F-met-puro was prepared with *E. coli* ribosomes. The reaction mixture contained in a total volume of 0.5 ml: 0.1 *M* Tris-HCl, pH 7.4; 0.075 *M* NH_4Cl; 0.01 *M* Mg acetate; 0.0005 *M* DTT; 0.0025 *M* guanosine 5′-triphosphate (GTP); 0.279 OD_{260} units of ApUpG; 0.001 *M* puromycin; 780 μμmoles of *E. coli* F-met-tRNA; and 20 OD_{260} units of *E. coli* ribosomes. The reaction mixture was incubated for 20 min at 30°, and the reaction was terminated by quick cooling and the addition of 1.5 ml of 0.1 *M* phosphate buffer, pH 8.0. To avoid oxidation of the methionine residue, oxygen was removed from the solution by evacuation, and all subsequent operations were carried out under nitrogen. The F-met-puro formed was extracted from the reaction mixture into 3 ml of freshly distilled ethylacetate. The resulting aqueous layer was re-extracted with 1.5 ml of ethylacetate, and the ethylacetate fractions were combined and evaporated to dryness. The resulting material (F-met-puro) was dissolved in 0.5 ml of 0.001 *M* Tris-HCl, pH 7.4. The yield of F-met-puro was 30–50% from F-met-tRNA; contamination by met-puro was less than 7%.

Preparation of N-terminal coliphage f2 coat fragment: The RNA from the sus 3 mutant of the f2 phage was used as template to direct the synthesis of the coat fragment F-met-ala-ser-asn-phe-thr in an *in vitro* protein-synthesizing system, as described by Webster *et al.*[13] A 0.5-ml reaction containing 3.6 OD_{260} units of sus 3 RNA and the selected radioactive amino acids was terminated by quick cooling after a 20-min incubation. The reaction was made 5% in trichloroacetic acid (TCA) by the addition of 0.05 ml of cold 50% TCA, and the precipitate was collected by centrifugation after 15 min at 0°. The supernatant was layered on a column composed of Bio-Rad AG 50W-X8 (1.3 × 7 cm) topped with Sephadex G-10 (1.3 × 14 cm) equilibrated with pH 1.9 buffer[4] containing 0.1% thiodiglycol. The column was developed with the same buffer; the first radioactive peak to elute was collected and concentrated *in vacuo* to 0.5 ml. It was layered on a Sephadex G-15 column (0.9 × 55 cm) equilibrated with 0.001 *M* Tris-HCl, pH 7.5, and run with the same buffer under a nitrogen atmosphere in order to avoid oxidation of the methionine residue of the coat fragment. The main radioactive peak to elute was the coat fragment. It was approximately 80–90% pure by electrophoresis at pH 1.9, 5.0, and 10, as described by Webster *et al.*[4]

Chemical deformylation and oxidation of the coat fragment: The *N*-terminal-formyl group of the coat fragment was removed by treatment at 90° with 0.5 *N* HCl for 10 min. The hexapeptide was purified by gel filtration through Sephadex G-10 in 0.001 *M* Tris-HCl, pH 7.4. The methionine residue of the coat fragment was oxidized by using performic acid as described by Nathans.[14]

Materials.—C^{14} and H^3-methionine, C^{14}-alanine, and H^3-formate were obtained from New England Nuclear Corp.; H^3-guanosine 5′-diphosphate (GDP) from Schwarz Bio-Research, Inc.; and ApU from Gallard-Schlesinger Chemical Mfg. Corp. THFA was supplied by General Biochemicals, Inc.; F-met, met-ala, ala-ser, ser-ala, phe-ser-thr by

Cyclo Chemical Corp.; puromycin dihydrochloride by Nutritional Biochemicals Corp.; and alkaline phosphatase by Worthington Biochemical Corp. T_1-RNase was a gift from Dr. Kenji Takahashi. Polynucleotide phosphorylase from *M. lysodeikticus* was purchased from P-L Biochemicals, Inc. and was purified by *O*-(diethylaminoethyl) (DEAE)-cellulose as described by Singer.[15] 5'-Guanylyl-methylene-diphosphonate (GMP-PCP) was obtained from Miles Chemical Co.; thiodiglycol from Pierce Chemical Co.; and Bio-Rad AG 50W-X8 from Bio-Rad Laboratories. Other chemicals were obtained as previously described.[10]

Results.—F-met-tRNA binding and F-met-puro formation: *B. subtilis* F-met-tRNA (prepared enzymatically with *B. subtilis* S-150) was bound to *B. subtilis* ribosomes charged with AUG in the presence of 5 mM Mg^{++} and GTP (Fig. 1). The addition of puromycin to the F-met-tRNA-AUG-ribosome complex resulted in the release of F-met-puro (Fig. 1). The formation of F-met-puro increased nearly linearly to 20 $\mu\mu$moles within 60 minutes, whereas the binding of F-met-tRNA to the same amount of ribosomes reached a plateau at about 3 $\mu\mu$moles within 20 minutes under the same conditions. This indicated a rebinding or a turnover of F-met-tRNA on the ribosomes after puromycin release. The rate of turnover depended on GTP concentration. As shown in Table 1, when the GTP concentration was increased, F-met-puro formation increased nearly proportion-

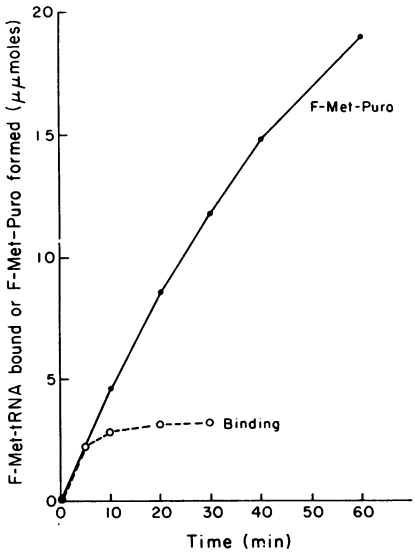

FIG. 1.—F-met-tRNA binding and F-met-puro formation. The complete reaction mixture contained in a total volume of 0.05 ml: 0.1 *M* Tris-HCl, pH 7.4, 0.005 *M* Mg acetate, 0.075 *M* NH_4Cl, 0.279 OD_{260} units of ApUpG, 0.0027 *M* GTP, 22 $\mu\mu$moles of *B. subtilis* F-met-tRNA, 0.001 *M* puromycin dihydrochloride, and 1.9 OD_{260} units of *B. subtilis* ribosomes. Puromycin was omitted in the binding experiments. The reaction was carried out at 24°. For assay, see *Methods*. Ethylacetate extraction at pH 8.0 was used for measuring F-met-puro formation.

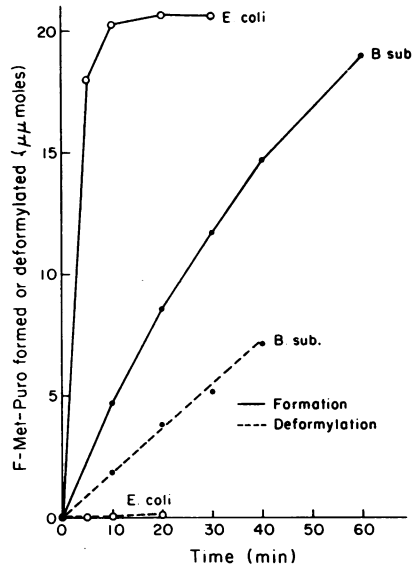

FIG. 2.—Formation and deformylation of F-met-puro. The reactions were carried out at 24° in the same reaction mixture as described in Fig. 1, except that in the experiments with *E. coli* ribosomes, equal amounts of *E. coli* ribosomes were substituted for *B. subtilis* ribosomes. For assay, see *Methods* and Fig. 1.

TABLE 1. *GTP, GMP-PCP effects on F-met-tRNA binding and puromycin release.*

GTP (mM)	GMP-PCP (mM)	Binding ($\mu\mu$moles)	Release ($\mu\mu$moles)
0	—	0.4	0.5
1.25	—	3.4	5.4
2.5	—	3.1	9.3
3.75	—	—	11.5
—	1.25	1.2	0.2
—	2.5	1.8	0.1
1.25	1.25	2.9	3.7
1.25	2.5	1.8	1.9

The reactions were carried out for 30 min at 24° in the same reaction mixture as described in Fig. 1, except that 21 $\mu\mu$moles of *E. coli* F-met-tRNA, GTP, or GMP-PCP at the concentrations shown in the table was used. Zero time controls were subtracted.

ally to the increase in concentration of GTP. There was, however, no effect on the binding of F-met-tRNA to ribosomes other than an initial stimulation.

As described by several investigators of the *E. coli* system,[16–19] GMP-PCP[02] can likewise substitute for GTP in the binding of F-met-tRNA to *B. subtilis* ribosomes. Only GTP, however, allows the formation of F-met-puro. GMP-PCP appears to inhibit the F-met-puro formation by competing with GTP.

Deformylase activity of B. subtilis ribosomes: Puromycin release with *B. subtilis* ribosomes resulted in the formation of both F-met-puro and met-puro (Fig. 2). *E. coil* ribosomes produced only F-met-puro. The formation of met-puro by *B. subtilis* ribosomes was a result of the deformylation of F-met-puro and not the deformylation of F-met-tRNA, since there was no loss of cold 7 per cent TCA-precipitable radioactivity when (H^3-formyl)-methionyl-tRNA was incubated with *B. subtilis* ribosomes under the same conditions. *B. subtilis* ribosomes were also unable to hydrolyze F-methionine. These results indicate that *B. subtilis* ribosomes carry an enzyme that removes the formyl group of F-met-puro. This enzyme is similar to the peptide deformylase reported to be present in both *E. coli* and *B. stearothermophilus* by Adams,[6] and Fry and Lamborg.[21]

Distribution of deformylase: The distribution of deformylase activity in various fractions of *B. subtilis* and *E. coli* was tested by using F-met-puro synthesized by *E. coli* ribosomes. As shown in Table 2, there was a significant amount of deformylase activity in all *B. subtilis* fractions, especially with the ribosomes. The *E. coli* fractions, however, showed almost no deformylase activity. Adams observed that the absence of deformylase in a cell-free protein-synthesizing system from *E. coli* was due to the instability of the enzyme to -SH reagents.[6] Therefore we prepared *E. coli* and *B. subtilis* supernatant and ribosome fractions in the absence of -SH reagents. Strong deformylase activity was present in both the S-150 and ribosome fractions from *E. coli*. The *E. coli* deformylase activity, however, was 50–100 per cent inhibited at 0.5–7.5 mM DTT, whereas the same concentration of DTT inhibited the *B. subtilis* deformylase activity no more than 10 per cent.

Hydrolysis of met-puro by an aminopeptidase: Table 2 shows that there was an aminopeptidase activity that further hydrolyzed met-puro to methionine and

TABLE 2. *Distribution of deformylase and peptidase.*

Enzyme sources	F-met-puro ($\mu\mu$moles) Deformylation	Further hydrolysis
B. subtilis S-150	0.4	0.4
B. subtilis ribosomes	1.1	0.2
B. subtilis S-150, *B. subtilis* ribosomes	1.4	1.1
E. coli S-150	0.0	0.1
E. coli ribosomes	0.2	0.1
B. subtilis ribosomes, *E. coli* ribosomes	1.1	1.1
B. subtilis ribosomes, *E. coli* S-150	1.3	1.0

The reactions were carried out for 10 min at 30° in a total volume of 0.05 ml containing 0.1 *M* Tris-HCl, pH 7.4, 0.075 *M* NH_4Cl, 0.005 *M* Mg acetate, 2.0 OD_{260} units of *B. subtilis* or *E. coli* ribosomes, 50 μg protein of *B. subtilis* or *E. coli* S-150, and 2.3 $\mu\mu$moles of F-met-puro. For assay, see *Methods*.

puromycin. *B. subtilis* S-150 produced 0.4 $\mu\mu$mole of met-puro by deformylation. The resulting 0.4 $\mu\mu$mole of met-puro was completely hydrolyzed to methionine and puromycin in the same reaction mixture. Interestingly, *B. subtilis* ribosomes, which have strong deformylase activity, made 1.1 $\mu\mu$moles of met-puro, but only 0.2 $\mu\mu$mole of this met-puro was further hydrolyzed. These results indicate that *B. subtilis* ribosomes contain deformylase but are quite free of the aminopeptidase. This aminopeptidase appears to be active with F-met-puro only after the formyl group has been removed by the deformylase.

B. subtilis S-150, *E. coli* S-150, and *E. coli* ribosomes under these conditions had low deformylase activity and therefore could not hydrolyze the peptide bond between methionine and puromycin. The addition of *B. subtilis* ribosomes, however, which carry deformylase but are quite free of peptidase activity, promoted deformylation and subsequently led to the hydrolysis of met-puro. The aminopeptidase activity was inhibited completely by 10^{-4} *M* EDTA. This inhibition could be reversed by 10^{-3} *M* Co^{++}, Mn^{++}, and Zn^{++}. A slight inhibition of the deformylase by this concentration of EDTA could be reversed by Mn^{++}. Similar observations have been made by Fry and Lamborg,[21] and by Adams[6] with the *E. coli* deformylase.

Hydrolysis of the N-terminal coat fragment of f2 phage: To test whether the possible physiological role of these enzymes is to sequentially remove the *N*-terminal portion of a newly synthesized *N*-formylmethionyl peptide, we used the *in vitro*-synthesized f2 phage coat fragment F-met-ala-ser-asn-phe-thr as substrate. The deformylase and aminopeptidase were separated from each other as described in *Methods*. The fragment labeled with C^{14}-methionine was incubated with both purified enzymes, and the radioactive products were identified by electrophoresis on cellulose acetate at pH 1.9 (Fig. 3). The partially purified deformylase catalyzed the formation of deformylated hexapeptide but produced only a small amount of free methionine. When the complete coat fragment was incubated with the partially purified peptidase, little, if any, degradation of the coat fragment occurred. In the presence of both enzyme preparations, however, a large peak corresponding to free methionine appeared. It is concluded that there are two separate enzymes: one a deformylase, which removes the formyl group from the *N*-formylated peptide, and another, an aminopeptidase, which

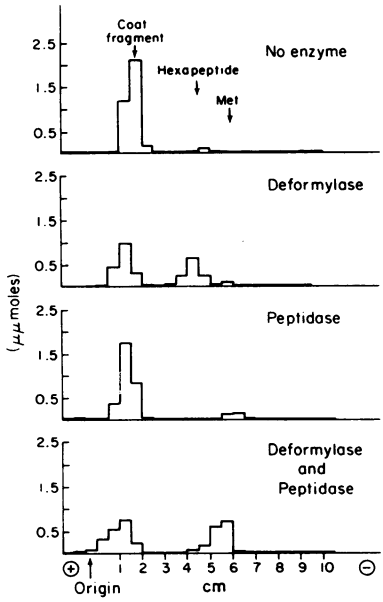

FIG. 3.—Enzymatic hydrolysis of coat fragment. The reactions were carried out for 40 min at 30° in a final volume of 0.022 ml containing 0.05 *M* Tris-HCl, pH 7.4, 4 μμmoles of coat fragment (C^{14}-methionine, 218 μc/μmole), 24 μg protein of deformylase and 60 μg protein of peptidase. The reactions were terminated by cooling and the products, in a 0.015-ml sample, were examined by cellulose acetate electrophoresis at pH 1.9, as described by Webster *et al.*[13] The coat fragment (formylated hexapeptide) was at the position marked 1–2 cm from the origin. Deformylated hexapeptide moved 4–5 cm. Methionine ran at about 5.5–6.5 cm. Alanine migrated to 7.5–8.5 cm.

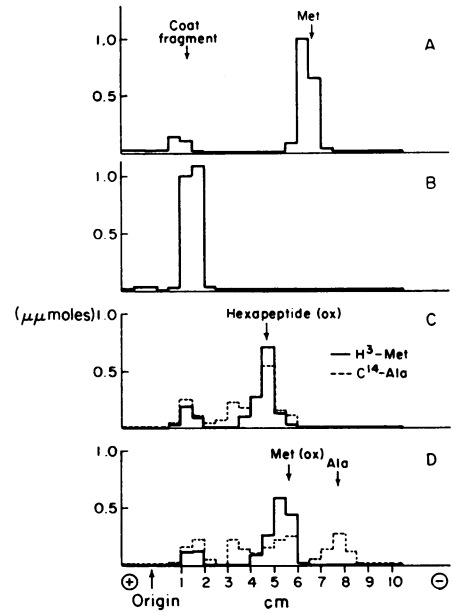

FIG. 4.—Substrate specificities of deformylase and aminopeptidase. The reactions and assay were the same as in Fig. 3.

(*A*) *B. subtilis* S-150 (100 μg protein containing deformylase and aminopeptidase) plus coat fragment (2.5 μμmoles, H^3-methionine 2920 μc/μM).

(*B*) *B. subtilis* S-150 plus oxidized coat fragment (2.5 μμmoles).

(*C*) Oxidized hexapeptide (2 μμmoles, H^3-methionine 2920 μc/μM, C^{14}-alanine 111 μc/μM).

(*D*) Oxidized hexapeptide pluse aminopeptidase (60 μg protein).

hydrolyzes the methionine from the peptide after the methionine amino terminus has been exposed. No enzymatic cleavage of formylmethionine from either the coat fragment or F-met-puro was observed.

Substrate specificities of deformylase and aminopeptidase: During the purification of formylated hexapeptide, we realized that oxidation of the methionine residue destroyed its ability to function as a substrate of deformylase. However, the use of thiodiglycol[22] and nitrogen in the course of peptide purification practically prevented the oxidation of the methionine residue, and the formyl group of the formylhexapeptide prepared in this manner could be almost completely removed by the deformylase (Fig. 4*A*). But after the methionine in the peptide was oxidized by performic acid, the deformylase could not remove the formyl group (Fig. 4*B*). This seems to indicate that the deformylase specifically reacts with the formylmethionine terminus of a peptide, especially since this defor-

mylase also does not deformylate F-ala-ser, F-ser-ala, or F-phe-ser-thr, nor does it deacetylate acetyl-met-ala. This specificity of *B. subtilis* deformylase resembles that observed for the *E. coli* peptide deformylase.[6]

If the formyl group was chemically removed from the oxidized formylhexapeptide labeled with H^3-met-C^{14}-ala (Fig. 4*C*), then the aminopeptidase could release free oxidized methionine from this hexapeptide (Fig. 4*D*). Furthermore, a small amount of alanine was also released, suggesting that the aminopeptidase may not be as specific as the deformylase for the methionine residue.

Discussion.—GTP-dependent *B. subtilis* F-met-tRNA binding to AUG-charged *B. subtilis* ribosomes was observed at 5 mM Mg^{++} concentration. GMP-PCP could support the binding to some extent. F-met-puro formation specifically required GTP, whereas GMP-PCP inhibited the reaction. Since Monro found that peptide synthetase does not require GTP,[23] it is most probable that the energy of GTP is required in a reaction between the binding of F-met-tRNA and peptide bond formation.[16, 17]

The amount of F-met-tRNA released by puromycin exceeded the amount of F-met-tRNA bound to the ribosomes, and the extent of the release was parallel to an increase in GTP concentration. This indicates that free tRNA must be removed from the ribosomes. The *B. subtilis* ribosomes contained not only initiation factors[24] but also G factor. This was determined by measuring the poly U-directed polyphenylalanine synthesis with *B. subtilis* ribosomes in the presence of purified *Pseudomonas* G and/or T factors (gifts of Drs. J. Lucas-Lenard and A-L. Haenni).[25] It is possible to speculate that F-met-tRNA first enters the acceptor site and is subsequently translocated into the donor site, with the concomitant hydrolysis of GTP possibly helping to remove the free tRNA.[26, 27]

The observations by Horikoshi and Doi[7] that methionyl-tRNA can be formylated by *B. subtilis* extracts and incorporated into protein *in vitro* suggest that this organism utilizes the same mechanism of chain initiation as that found in *E. coli*. Our observations that F-met-tRNA is able to bind to AUG-charged ribosomes and form F-met-puro on addition of puromycin support this hypothesis. Since approximately 80 per cent of the *N*-terminal residues of *B. subtilis* proteins are alanine and 10 per cent are methionine,[8] there must exist some mechanism for removing the formyl group and the methionine residues from the nascent proteins. We have described a deformylase that appears to be associated with the *B. subtilis* ribosomes and that is able to remove the formyl group from formylmethionine peptides but not from formylmethionine or formylmethionyl-tRNA. It is specific for methionine since formyl peptides containing oxidized methionine, alanine, serine, or phenylalanine on the *N*-terminus are inactive as substrates. It is also specific for the formyl group since acetyl-met-ala is inactive as a substrate. This specific deformylase is very similar in activity to the peptide deformylase described for *E. coli* and *B. stearothermophilus* by Adams.[6] However, the *B. subtilis* deformylase is much more resistant to thiols, thus explaining its strong activity in extracts.

The specificity of the deformylase for methionine and the absence of any activity capable of removing formylmethionine as a unit from the protein chain suggest that formylmethionine is removed from *B. subtilis* in two steps as pro-

posed by Capecchi[5] and Adams[6] for *E. coli:* first, the formyl group is removed by the deformylase and then methionine is removed by another enzyme. This proposed enzyme presumably would be a methionine-specific aminopeptidase. We have found an aminopeptidase in both *E. coli* and *B. subtilis* that is capable of removing methionine from the *N*-terminus in a peptide only if the amino group of the peptide is free. Further investigations will determine whether this aminopeptidase has the specificity needed to account for the observed amino terminal residues of *E. coli* and *B. subtilis* proteins.

Summary.—F-met-tRNA was able to bind to ApUpG-charged *B. subtilis* ribosomes and form F-met-puro by the addition of puromycin. The role of GTP and GMP-PCP in these reactions was discussed. A *B. subtilis* deformylase was described that was specific for the formyl group and *N*-terminal methionine of a peptide. A *B. subtilis* aminopeptidase activity was also described.

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[1] Marcker, K. A., and F. Sanger, *J. Mol. Biol.*, **8**, 835 (1964).
[2] Clark, B. F. C., and K. A. Marcker, *J. Mol. Biol.*, **17**, 394 (1966).
[3] Adams, J. M., and M. R. Capecchi, these PROCEEDINGS, **55**, 147 (1966).
[4] Webster, R. E., D. L. Engelhardt, and N. D. Zinder, these PROCEEDINGS, **55**, 155 (1966).
[5] Capecchi, M. R., these PROCEEDINGS, **55**, 1517 (1966).
[6] Adams, J. M., *J. Mol. Biol.*, **33**, 571 (1968).
[7] Horikoshi, K., and R. H. Doi, *Arch. Biochem. Biophys.*, **122**, 685 (1967).
[8] Horikoshi, K., and R. H. Doi, *J. Biol. Chem.*, **243**, 2381 (1968).
[9] Leder, P., and H. Bursztyn, *Biochem. Biophys. Res. Commun.*, **24**, 233 (1966).
[10] Takeda, M., and F. Lipmann, these PROCEEDINGS, **56**, 1875 (1966).
[11] Ochoa, S., personal communication.
[12] Nirenberg, M., and P. Leder, *Science*, **145**, 1399 (1964).
[13] Webster, R. E., D. L. Engelhardt, N. D. Zinder, and W. Konigsberg, *J. Mol. Biol.*, **29**, 27 (1967).
[14] Nathans, D., *J. Mol. Biol.*, **13**, 521 (1965).
[15] Singer, M. F., in *Procedures in Nucleic Acid Research*, ed. G. L. Cantoni and D. R. Davies (New York: Harper and Row, 1966), p. 245.
[16] Hershey, J. W. B., and R. E. Thach, these PROCEEDINGS, **57**, 579 (1967).
[17] Anderson, J. S., J. E. Dahlberg, M. S. Bretscher, M. Revel, and B. F. C. Clark, *Nature*, **216**, 1072 (1967).
[18] Ohta, T., S. Sarkar, and R. E. Thach, these PROCEEDINGS, **58**, 1638 (1967).
[19] Allende, J. E., and H. Weissbach, *Biochem. Biophys. Res. Commun.*, **28**, 82 (1967).
[20] Hershey, J. W. B., and R. E. Monro, *J. Mol. Biol.*, **18**, 68 (1966).
[21] Fry, K. T., and M. R. Lamborg, *J. Mol. Biol.*, **28**, 423 (1967).
[22] Moore, S., and W. H. Stein, *J. Biol. Chem.*, **211**, 893 (1954).
[23] Monro, R. E., *J. Mol. Biol.*, **26**, 147 (1967).
[24] Salas, M., M. B. Hille, J. A. Last, A. J. Wahba, and S. Ochoa, these PROCEEDINGS, **57**, 387 (1967).
[25] Lucas-Lenard, J., and F. Lipmann, these PROCEEDINGS, **55**, 1562 (1966).
[26] Nishizuka, Y., and F. Lipmann, *Arch. Biochem. Biophys.*, **116**, 344 (1966).
[27] Seeds, N. W., and T. W. Conway, *Biochem. Biophys. Res. Commun.*, **23**, 111 (1966).