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Isolation and point of action of a factor from *Escherichia coli* required to reconstruct translation

(ribosomes/translation initiation/translation propagation/translation termination/protein synthesis factor)

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ABSTRACT **To study the mechanism of translation we have attempted to reconstruct the process from purified components. Protein synthesis was programmed by the RNAs of wild-type or amber mutants of bacteriophages f2 or MS2. Translation programmed by MS2 or f2am3 RNA does not occur using ribosomes, precharged aminoacyl-tRNAs, and the sum of the purified proteins involved in initiation (initiation factors; IF-1, IF-2, and IF-3), propagation (elongation factors; EF-Tu, EF-Ts, and EF-G) and termination (release factors; RF-1 or RF-2) of protein synthesis. The requirement for a protein called W was demonstrated. Protein W was purified free of all translation factors, activating enzymes, and other proteins such as the RR, "rescue," and EF-P implicated in translation. The stimulation of propagation by W depended on the position of the amino acid residue to be added in the synthesis of the NH_2-terminal hexapeptide of the coat protein. In the reconstructed system, with the sum of all translation factors but in the absence of W, only dipeptides and smaller quantities of tripeptides were synthesized under the direction of f2am3 RNA. W stimulated the synthesis of the hexapeptide, fMet-Ala-Ser-AspNH_2-Phe-Thr directed by this RNA. In addition, W stimulated ejection of non-cognate tRNAs that bind to ribosomal particles.**

Understanding of the mechanism by which the genetic code is deciphered depends on reconstruction of the translation process from purified components. A major step in accomplishing this task was achieved by Traub and Nomura (1) and Nierhaus (2) in the assembly of both the ribosomal subunits from rRNA and ribosomal proteins. Translation also requires the participation of 60 tRNAs, 20 activating enzymes, and proteins that promote initiation (initiation factors; IF-1, IF-2, IF-3), propagation (elongation factors; EF-Tu, EF-Ts, EF-G) and termination (release factors; RF-1, RF-2, RF-3) (3, 4).

In addition, several proteins have been reported to stimulate partial reactions of translation. One of these, EF-P, stimulates peptide-bond synthesis between fMet-tRNA and either analogues of aminoacyl-tRNAs, such as puromycin, or certain CCA amino acids (e.g., glycine or leucine) but not others (5–7). Protein X specifically stimulates synthesis programmed by certain synthetic templates other than poly(U) (8). According to one report, there is a factor that stimulates ejection of $tRNA_f^{Met}$ from ribosomes (9). Finally, a series of activities has been described that is directly or indirectly implicated in the translation process. These include fMet-tRNA hydrolase (10), peptidyl-tRNA hydrolase (11, 12), and the RR factor (13) that releases mRNA from ribosomes preventing reinitiation of translation (14). Study of conditionally lethal mutants defective in translation permitted identification of still another protein, "rescue," which acts in an undefined step of the ribosomal cycle (15–18).

Reconstruction of translation from a DNA-directed system, ribosomes, and >30 proteins including several transcription factors, 20 activating enzymes, initiation, propagation, and termination factors revealed that the protein RR as well as activating enzymes, the transformylase, and several translation factors are essential for protein synthesis (3, 19–21). However, the requirements for several known and perhaps new components could not be scored, presumably because of the complexity of the system (20).

To study the mechanism, it is essential to simplify the number of proteins required to catalyze protein synthesis. To do this, we have used a mixture of aminoacyl-tRNAs containing the labeled initiator tRNA and programmed each facet of synthesis with RNAs of either MS2, f_2, or amber mutants of these bacteriophages. The bulk of the product synthesized by each of these bacteriophage mRNAs is the coat protein. Because the sequence of the coat protein is known, the use of appropriate amber mutants in the coat gene permits the study of events at specific sites on the message; analysis of the NH_2-terminal products defines each intermediate event in the protein synthesis reaction.

Here we report that the sum of the highly purified initiation and propagation proteins does not permit reconstruction of translation from fMet-tRNA and precharged aminoacyl-tRNAs programmed by MS2 or f2am3 RNA. Addition of protein W, isolated from ribosomal eluates, enhances synthesis programmed by MS2 RNA. Protein W is required for translation of the am3 mutant of f2 RNA and is indispensable to *in vitro* reconstruction of protein synthesis. We propose that protein W prevents the spurious entrance of deacylated tRNA into the particles.

MATERIALS AND METHODS

[^{35}S]Methionine (829 or 550 Ci/mmol; 1 Ci = 37 GBq), [^{3}H]serine (29.6 Ci/mmol), [^{3}H]lysine (60 Ci/mmol), and [^{32}P]ATP (700 Ci/mmol) were purchased from New England Nuclear. Crude tRNA from *Escherichia coli* B was bought from Amersham and Searle. *E. coli* K-12 midlogarithmic phase cells, $tRNA^{Phe}$, and poly(U) were purchased from Miles.

f[^{35}S]Met-tRNA containing 20 unlabeled aminoacyl-tRNAs and ribosomes from *E. coli* Q13 or MRE 600 were prepared as described (10). Ribosomes were analyzed by two-dimensional polyacrylamide gel electrophoresis as described by Kaldschmidt and Wittmann (22). MS2 or f2 bacteriophages or the amber mutant f2am3 were grown on *E. coli* strain K37 (23) and their RNA was isolated as described (24). Met, Met-Ala, and Met-Ala-Ser were purchased from

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Abbreviations: IF, initiation factor; EF, elongation factor; RF, release factor.

Schwarz/Mann and were chemically formylated (25). N-blocked amino acids and peptides were visualized as described (26).

Purification of Known Protein Synthesis Factors. The initiation factors IF-1 and IF-2 were assayed and purified by the method of Suttle *et al.* (27) or Hershey *et al.* (28). IF-3 was assayed using saturating levels of IF-1 and IF-2, S100 from *E. coli* MRE 600, or Q13 and Q13 ribosomes and MS2 RNA (28). The propagation proteins EF-Tu, EF-Ts, and EF-G were assayed and isolated by the procedures of Gordon *et al.* (29) or by the method of Arai *et al.* (30). In some experiments, homogeneous EF-Tu, EF-Ts, and EF-G (donated by K. Nierhaus) were used. EF-P was purified as described (6). Rescue factor was purified to stage V or VI (18) using as an assay the complementation of extracts from the mutant N4316 at 40°C–44°C as described (18). Release factors RF-1 and/or RF-2 were purified as described by Ganoza *et al.* (31).

Purification of Component W. The 1.0 M NH_4Cl wash from *E. coli* K-12 ribosomes (from 200–400 g of cells) was concentrated by addition of 80% ammonium sulfate (pH 7.0). The precipitate was dissolved in buffer A [10 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.5/1.0 mM dithiothreitol/0.5 mM EDTA/10% (vol/vol) glycerol], dialyzed, and chromatographed on a DEAE-cellulose (Whatman DE 32) column (2.6 × 40 cm). Approximately 2 mg of protein per ml of column volume was loaded and the column was first washed with 240 ml of buffer A/0.05 M NH_4Cl, and then eluted with a 0.05–0.35 M NH_4Cl gradient in buffer A (240 ml in each reservoir). Component W eluted with IF-2 at ≈0.2 M NH_4Cl. The active fraction was concentrated on Amicon P-30 filters, and dialyzed against buffer B [50 mM Tris·HCl, pH 7.4/1 mM dithiothreitol/0.5 mM EDTA/10% (vol/vol) glycerol] and chromatographed on a cellulose phosphate (Whatman P-11) column (1.6 × 12 cm) equilibrated with buffer B. All the component W activity was recovered by washing the columns with 120–200 ml of buffer B/0.05 M NH_4Cl. All the IF-2 activity was retained on cellulose phosphate and eluted with a gradient of 0.05–0.60 M NH_4Cl. The P-11 wash fraction (usually 3–4 mg/ml) was diluted 1:5 in 10 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.5/1 mM dithiothreitol and applied to a hydroxylapatite column (0.9 × 15 cm) equilibrated with the same buffer. The column was eluted batchwise with K_2HPO_4/KH_2PO_4 buffer, pH 7.5, and the component W activity was eluted with 70 mM K_2HPO_4/KH_2PO_4 buffer. After concentration, W was dialyzed against buffer B/50 mM NH_4Cl and 19 mg of the fraction was applied to a Sephacryl G-200 column (94 × 1.6 cm) equilibrated with 10 mM Tris·HCl, pH 7.4/50 mM NH_4Cl/1 mM dithiothreitol. W eluted from these columns with an apparent molecular weight of 60,000.

Reconstruction Assay. The reconstruction assay from aminoacyl-tRNAs (one-step transfer reactions) contained, in a vol of 55 μl, 10.9 mM $MgCl_2$/35 mM Tris buffer, pH 7.4/32 mM NH_4Cl/0.5 mM GTP/1.0 mM ATP/9 mM dithiothreitol/100 μg of ribosomes/10 μg of MS2 or f2 RNA or f2am3 RNA, and generally from 1.2 to 3.5 μg of IF-1, 1.3 to 1.4 μg of IF-2, 2.1 to 2.5 μg of IF-3, 0.3 μg of EF-Tu, 0.1 to 0.5 μg of EF-Ts or 2.6 to 7 μg of EF-T, 0.9 to 3.1 μg of EF-G, ≈5 μg of release factors, "rescue" protein, EF-P, and component W, and 100,000 to 240,000 dpm of f[^{35}S]Met-tRNA^{Met}, and all other unlabeled aminoacyl-tRNAs. Incubations were for 30 min at 35°C, unless otherwise specified. Two-step transfer reactions (synthesis from amino acids) contained the same components as the one-step transfer reaction and ≈11 × 10^6 [^{3}H]lysine, 0.09 mM unlabeled amino acids, and 2.8 mM pyruvate, 2.5 μg of pyruvate kinase, and 1.5 μg of Ca^{2+} leucovorin and either purified activating enzymes (0.1–0.2 μg of each, a gift of H. Weissbach, Roche Institute, NJ) and unlabeled fMet-tRNA or the S100 fraction. The two-step transfer reaction was used routinely to monitor the purification of component W. Reactions were stopped with 10% Cl_3CCOOH, heated 15 min at 90°C, and filtered through millipore (0.45-μm pore size) or glass fiber filters.

Hexapeptide Assay. The formation of the acid-soluble hexapeptide and other peptides made under the direction of f2am3 RNA was assayed essentially as described by Capecchi (32) using either f[^{35}S]Met-tRNA and each unlabeled aminoacyl-tRNA or [^{3}H]Thre-tRNA containing all of the other unlabeled aminoacyl-tRNAs except that the electrophoresis was for about 2.5 hr on 1 mm Whatmann paper at 40 V/cm on a cooled Buchler high voltage chamber.

Radioactivity was determined as described (18). Protein was estimated by the method of Lowry *et al.* (33).

RESULTS

The simplest partial reconstruction assay uses IFs purified from ribosomes and the S100 fraction as a source of activating enzymes, the Met-tRNA^{Met} transformylase, and all other factors needed for protein synthesis (for reviews, see refs. 3 and 34). Using this assay, we find that the purified IFs do not fully reconstruct synthesis. As shown in Fig. 1*A*, little synthesis of MS2-directed products was detected with IF-1 and IF-2. IF-3 stimulated synthesis ≈4-fold, as expected (27, 28, 35). Addition of the protein fraction that did not adsorb to phosphocellulose greatly stimulated protein synthesis (see Fig. 1*A*). This observation was used as the basis for an assay to purify the protein tentatively designated as "W" that stimulates protein synthesis.

Autoradiograms of $NaDodSO_4$/polyacrylamide gels used to analyze the products of the synthesis stimulated by component W are shown in Fig. 1*B*. These experiments show that omission of IF-3 or W from the complete system results in a drastic decrease in synthesis of the coat protein of MS2 RNA. Further addition of 20 purified activating enzymes failed to substitute for W (Fig. 1*B*, lane 3). The fact that W cannot be replaced by the ribosome-associated initiation factors IF-1, IF-2, and IF-3; by ribosomal protein S1 or the soluble proteins EF-Tu, EF-Ts, EF-G, EF-P; or by rescue is summarized in Fig. 2.

Properties of Component W. Routinely, 30% of component W activity is found in the S100 extract and the remaining 70% is found in the ribosomal wash. W activity purified as described in *Materials and Methods* has an $s_{20,w}$ value of 4.1 ± 0.15, which corresponds to an approximate molecular weight of 60,000. The major band seen on $NaDodSO_4$ gel electrophoresis of the most highly purified W preparations migrates as a protein of M_r 62,000. Purified W has no IF-1, IF-2, IF-3, EF-Tu, EF-Ts, EF-G, RF-1, RF-2, "rescue", EF-P, activating enzyme, or transformylase activity. The factor contained <0.5% S1 or S1A and did not cross-react with antibodies to these proteins or to antibodies against EF-Tu, EF-Ts, EF-G, or the RR protein (data not shown).

Minimum Components Required for Reconstruction. To simplify the requirements for translation, we examined the MS2-dependent synthesis programmed by precharged aminoacyl-tRNAs and labeled f[^{35}S]Met-tRNA. Without W, the sum of each of the highly purified soluble proteins, IF-1, IF-2, IF-3, EF-Tu, EF-Ts, and EF-G, stimulated each partial reaction of translation, but gave at most 19% of the MS2-dependent translation observed with crude extracts (Fig. 3). This value was not appreciably altered by addition of RF-1 and/or RF-2, or EF-P. The "rescue" protein did stimulate synthesis slightly but reproducibly, but none of these proteins substituted for W in the simplest reconstruction assay of MS2-directed translation (data not shown).

Analysis of NH_2-Terminal Products of Synthesis Programmed by f2am3 RNA. The NH_2-terminal products of protein synthesis can be readily analyzed with f2am3 RNA,

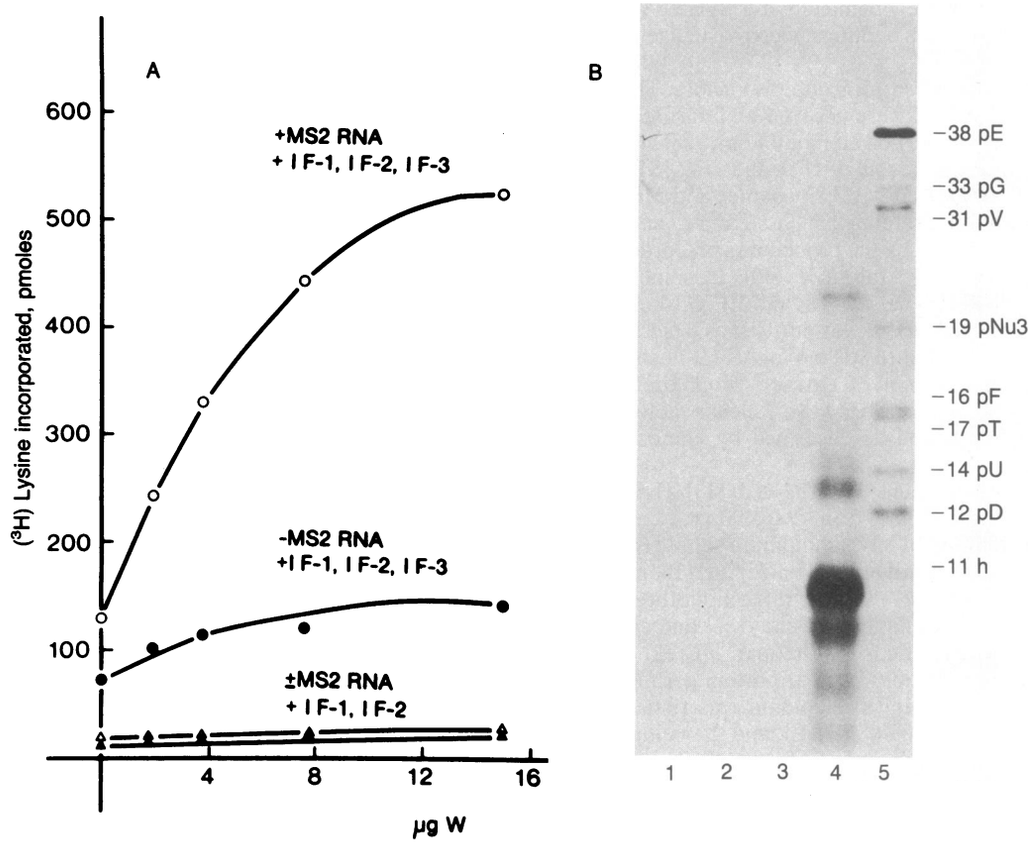

FIG. 1. (A) Stimulation of MS2-dependent protein synthesis by component W. Two-step reconstruction reactions are as described in *Materials and Methods*. The W preparation was purified through the cellulose phosphate step. (B) Analysis of labeled MS2-directed synthesis products on $NaDodSO_4$/polyacrylamide gels (40). Reaction mixtures were as described in *Materials and Methods* and in A except that 25 μl of the reactions contained 4.6 pmol of [^{35}S]methionine (829 Ci/mmol). After incubation, the reactions were treated with RNase, concentrated (17), and electrophoresed on 15% polyacrylamide gels containing $NaDodSO_4$ (40). The gels were dried and exposed to x-ray film for 4 days (17). Lanes: 1, reactions without IF-3; 2, reactions without W; 3, reactions without W and with 20 activating enzymes and fMet-tRNA; 4, the complete system; 5, ^{35}S λ late protein marker (400 × 10^3 dpm) (17).

which directs the synthesis and premature termination of the NH_2-terminal hexapeptide (fMet-Ala-Ser-AspNH_2-Phe-Thre) of the virion's coat protein. Fig. 4 shows a typical electrophoretic analysis of these products labeled with f[^{35}S]-Met-tRNA and each unlabeled aminoacyl-tRNA. The experiment shows that, without W, the major product synthesized with all the known initiation and propagation factors is fMet-Ala. Much less of the pentapeptide and about one-half of the tripeptide are synthesized. Addition of W decreases the level of the dipeptide and stimulates synthesis of longer products. In keeping with this, we find that W stimulates the incorporation of [^{3}H]Ala-tRNA only 39% while stimulating incorporation of [^{3}H]Ser-tRNA 110% and [^{3}H]Phe-tRNA or [^{3}H]Thre-tRNA 220% over reactions not containing W (data not shown).

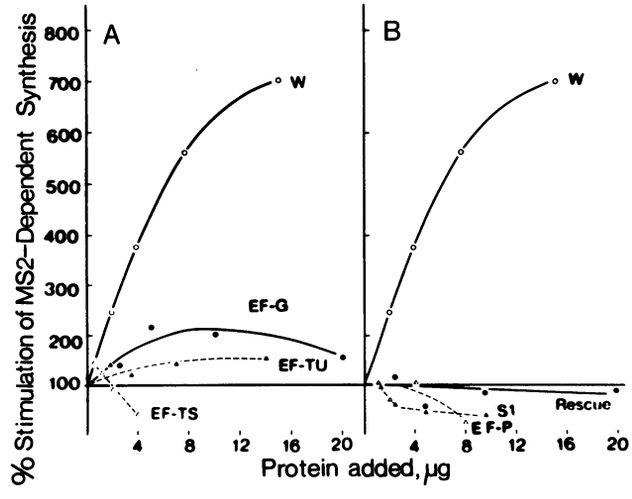

FIG. 2. Inability of known protein synthesis factors to substitute for W. Each translation factor, including W, was assayed. [^{3}H]Lysine (29 pmol) was incorporated (100% stimulation) in reactions with MS2 RNA without added W. These incubations were supplemented with the indicated levels of W, EF-G, EF-Tu, EF-Ts, EF-G (A); S_1, "rescue," and EF-P (B) purified as described in *Materials and Methods*.

The fact that chain extension beyond the dipeptide stage proceeds inefficiently without W suggests that the translocation step of translation is directly or indirectly affected by this protein. However, the translocase, EF-G, was unable to substitute for protein W (Fig. 4) and two mutants defective in EF-G contained saturating levels of protein W (data not shown).

Since deacylated tRNA binds spontaneously to ribosomes (Fig. 5), we asked whether W prevents this unspecific codon-independent attachment of deacylated tRNA. In this experiment, chain growth was limited by use of a mutant of EF-G, which arrests synthesis at the dipeptide stage (4). The binding of the third NH_2-terminal amino acid of the coat protein of MS2 RNA, [^{3}H]Ser-tRNA, and of deacylated [^{32}P]-$tRNA^{Phe}$ was monitored. W was found to stimulate binding of [^{3}H]Ser-tRNA in the presence of EF-Tu, EF-Ts, and EF-G (Fig. 5). W prevents the binding of native or deacylated [^{32}P]$tRNA^{Phe}$ to ribosomes.

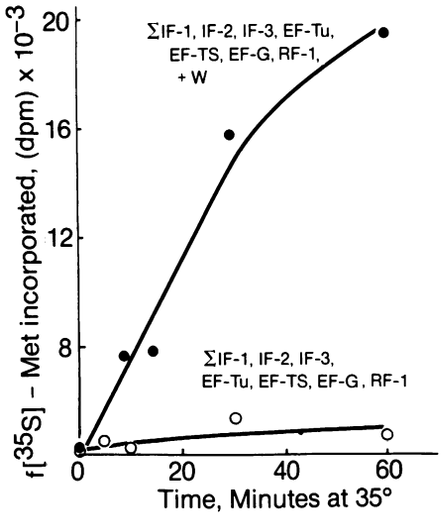

FIG. 3. Kinetics of reconstruction of protein synthesis. Incubations were as described in *Materials and Methods* for one-step transfer reactions. Aliquots of 40 μl were withdrawn at the indicated times to assay for acid-insoluble radioactivity. Incubations (160 μl) contained 2 μg each of IF-1, IF-2, and IF-3, 1.0 μg of EF-Tu, 8 μg of EF-Ts, 4 μg of EF-G, 12.5 μg of RF-1, 15 μg of MS2 RNA, 81,000 dpm (0.7 pmol) of f[^{35}S]Met-tRNA^{Met}, 150 μg of Q13 ribosomes, and, where indicated, 14 μg of W purified through the hydroxylapatite step.

We conclude that W acts on a previously undefined event in the propagation of protein synthesis.

DISCUSSION

We report that the sum of the "soluble" proteins that support chain initiation, propagation, and termination in model reactions of translation is essentially inactive in promoting the translation of the MS2 phage RNA template or the RNA of an amber mutant of the closely related phage f2. We find that specific MS2-directed products can only be observed when protein W is added to the reactions.

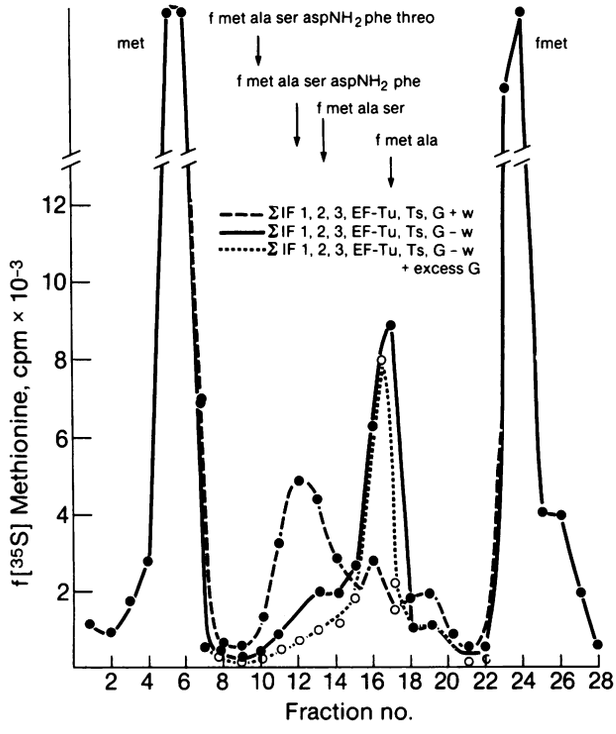

FIG. 4. Effect of W on the products of the reconstructed synthesis reactions programmed with f2am3 RNA. One-step transfer reactions were as described in *Materials and Methods*. After incubation, reaction mixtures were treated with 1.75 M NH_2OH for 30 min at 35°C, and acid-soluble N-blocked peptides were analyzed. To quantitatively convert the hexapeptide into pentapeptide, the reactions were then treated with 50 μg of chymotrypsin in 14 mM EDTA for 2 hr at 24°C.

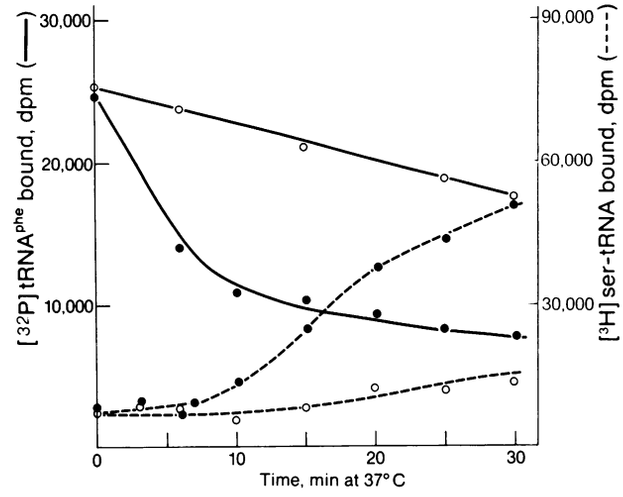

FIG. 5. Effect of W on the binding of [^{32}P]tRNA^{Phe} and [^{3}H]Ser-tRNA to ribosomes programmed with MS2 RNA. Components for the binding reaction were the same as described for the two-step reconstruction reaction except that ≈23 μg of S100 from the *E. coli* K-12 strain G1 temperature-sensitive in EF-G (6) was used as a source of activating enzymes and factors. Each 25 μl also contained 67 μg of Q13 ribosomes, 0.27 μg of IF-1, ≈1 μg of IF-2, 6.3 μg of IF-3, 35 pmol of [^{3}H]serine, 1.7 × 10^5 dpm of [^{32}P]tRNA^{Phe} [kinased and isolated as described (36)], and, where indicated, 7.1 μg of W purified through the cellulose phosphate step and 0.6 μg of EF-G. Reaction mixtures (150 μl) were incubated at 37°C and 15-μl aliquots were removed to binding buffer at the indicated times and filtered on millipore filters (37). [^{3}H]Ser-tRNA bound to ribosomes with added W (●—●) or without added W (○—○); [^{32}P]tRNA^{Phe} bound to ribosomes with added W (●—●) or without added W (○—○).

Protein W was purified free of ribosomal proteins, activating enzymes, Met-tRNA transformylase, and all known translation factors including RR, EF-P, and the "rescue" protein as well as enzymes involved in the synthesis of ATP or GTP.

Analysis of the products synthesized in the reconstructed system suggests that W stimulates protein chain propagation. Using each of the labeled aminoacyl-tRNAs that occur at the NH_2-terminus of the coat protein synthesized with either MS2 or f2am3 RNA, we find that binding of fMet-tRNA is not affected by protein W. Stepwise analysis of the hexapeptide labeled with each subsequent amino acid suggests, instead, that the degree of stimulation of protein synthesis by W depends approximately on the type and number of residues in the nascent peptide.

It has been reported that dipeptidyl-tRNA unbinds from ribosomes (8, 21), whereas longer nascent chains remain bound to ribosomes (21). The stability of the nascent peptidyl-tRNA may be affected by the nature of the tRNA–template combination. Thus, polyphenylalanyl-tRNA synthesized with poly(U) remains stably bound to ribosomes, whereas polylysyl-tRNA synthesized with poly(A) is equally distributed in the soluble and ribosomal fractions (8).

Protein W, as was the case for the partially purified factor X previously reported (8), is not required for chain extension of polyphenylalanine but is required for synthesis from other artificial templates such as poly(A).

W exerts its strongest stimulatory effect in pentapeptide synthesis and, in conjunction with EF-G, also promotes tripeptide synthesis (Figs. 4 and 5). The translocation event requires several steps, including (*i*) release of tRNA from

ribosomes after peptide-bond synthesis has occurred, (*ii*) movement of the nascent peptide from the ribosomal P to the A site, (*iii*) recognition of the next codon in the mRNA, and (*iv*) possible ejection of tRNA from the ribosomal E site (3, 4, 38, 39). W does not stimulate ejection of $[^{32}P]tRNA_f^{Met}$ from ribosomes. Since W is not required for polyphenylalanine synthesis, we think it unlikely that it would be involved in step *ii* or *iii* of the mechanism.

It has been reported that EF-G stimulates unbinding of deacylated tRNA (3, 4). This action of EF-G occurs in the presence of excess deacylated tRNA (38, 39). It has been proposed that deacylated tRNA is not released from the P site but is first translocated to the E site and that the EF-Tu-dependent binding of aminoacyl-tRNA to the A site stimulates the exit of tRNA from the ribosomal E site (38, 39). We observe that W (and also EF-Tu, but not EF-G; data not shown) prevents the attachment of deacylated tRNA to ribosomes (Fig. 5). Although the reaction is observed with EF-Tu, the fact that protein W is free of EF-Tu and does not cross-react with anti-EF-Tu suggests an alternate mechanism for this reaction, which is promoted by protein W.

Protein W prevents the binding of deacylated or periodate-treated $[^{32}P]tRNA^{Phe}$, further suggesting that, unlike EF-G or EF-Tu, this protein may remove from ribosomes tRNA that is not necessarily a product of peptide-bond synthesis. Unbinding of tRNA precedes tripeptide synthesis, suggesting that W stimulates propagation by removing noncognate deacylated tRNA from ribosomes. This event may also stimulate the more stable attachment of the nascent chain on the ribosome. Alternatively, the binding of tRNA to ribosomes and its unbinding promoted by W could regulate the onset of propagation in a positive manner.

Deacylated tRNA has been found in active polysomes of eukaryotic and prokaryotic cells (for review, see ref. 39) and it has been proposed that its occurrence in the ribosomal E site serves an essential, as yet undefined, step of protein synthesis. The approach reported here to monitor stepwise translation using highly purified proteins coupled to genetic manipulation may help define this and other complex reactions that regulate the protein synthetic process.

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