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Analysis of the Two Steps in Polypeptide Chain Initiation Inhibited by Pactamycin†

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ABSTRACT: Earlier work has shown that the inhibition by pactamycin (PM) of polypeptide chain initiation in reticulocyte extracts is associated with (1) a defect in the joining of the 60S subunit to the smaller initiation complex to form an 80S complex ("joining reaction") (Kappen, L. S., Suzuki, H., and Goldberg, I. H. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 22) and (2) a block after the synthesis of the initial dipeptide (Kappen, L. S., and Goldberg, I. H. (1973), *Biochem. Biophys. Res. Commun. 54,* 1083). The relative contributions of these two effects to the action of PM and their relationship to one another were evaluated in a system employing sparsomycin that permits both initiation at a certain number of initiation sites and limited oligopeptide formation without termination and release. The degree to which PM blocks the "joining reaction" and leads to the accumulation of 48S initiation complexes that either remain free or are bound to polysomes without the corresponding 60S subunit ("half-mers") was estimated by treatment of polysomes with RNase. Met-$tRNA_f^{Met}$ binding factors are required to stabilize the RNase-generated 48S complexes. Under conditions where the initiation factor required for the "joining reaction" functions catalytically, presumably by cycling on and off initiation complexes, PM usually inhibits 80S complex formation 50–70%. Where "joining" is not limiting (presence of at least stoichiometric amounts of joining factor or high Mg^{2+} concentration) PM leads to the maximal accumulation of the initial dipeptide, Met-Val, in the P-site on the ribosome, indicating a block in a subsequent step in elongation. Binding studies with [^{3}H]PM and the inability of PM to inhibit elongation of preformed Met-Val indicate that PM must interact with the ribosomes at an early stage of initiation. Taken together these data are compatible with the suggestion that PM does not interfere with the ribosomal "joining reaction" per se, but prevents the release and reuse of the joining factor, and in so doing blocks a step in elongation after formation of the initial dipeptide and its translocation to the P-site on the ribosome.

Low levels of the antibiotic pactamycin (PM),[1] a potent inhibitor of protein synthesis in intact cells and cell-free extracts of eucaryotes (Colombo et al., 1966; Felicetti et al., 1966; Macdonald and Goldberg, 1970), preferentially inhibit the initiation of polypeptide chains (Macdonald and Goldberg, 1970; Stewart et al., 1971; Lodish et al., 1971; Goldberg et al., 1973; Ayuso and Goldberg, 1973). We have previously reported (Kappen et al., 1973; Goldberg et al., 1973; Goldberg, 1974) that in a fractionated system (salt-washed ribosomes and crude initiation factors) derived from rabbit reticulocytes PM does not inhibit the factor- and GTP-dependent binding of [^{35}S]Met-$tRNA_f^{Met}$ to the 40S subunits, but at 2 mM Mg^{2+} prevents the formation of a stable, functional, larger (80 S) initiation complex. This results in the accumulation of smaller initiation complexes (48 S) and hybrid ("one and one-half-mer") structures, composed of a monosome and an attached [^{35}S]Met-$tRNA_f^{Met}$ bearing a 40S subunit at the mRNA initiation site. Under these conditions there appears to be a block in the joining of the 60S ribosomal subunit to the smaller initiation complex. On the other hand, at 5 mM Mg^{2+} 80S initiation complex formation can take place in the presence of PM. Similarly, addition to excess of a factor required for the normal joining of the 60S subunit to the 48S ribosomal initiation complex reverses PM inhibition of 80S complex formation and methionylpuromycin synthesis (Suzuki and Goldberg, 1974). We have further shown (Kappen and Goldberg, 1973) that in reticulocyte lysate containing PM about 50% of the radioactivity derived from [^{35}S]Met-$tRNA_f^{Met}$ that is bound to monosomes and oligosomes is in the form of methionylvaline, the initial dipeptide of globin chains, indicating the existence of a block after the forma-

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[1] Abbreviations used are: PM, pactamycin; ATA, aurintricarboxylic acid.

tion of the first peptide bond. Thus, depending upon the particular experimental conditions in vitro, PM exerts two effects, both resulting in inhibition of polypeptide synthesis.

It is thus important to determine the relative contributions of these two effects to the action of PM in inhibition of protein synthesis and to determine whether the two effects are interrelated. In addition, we want to know if the specificity of PM for polypeptide chain initiation is due to the availability of ribosomal binding sites only at this or a preceding stage.

Materials and Methods

Reticulocytes, Lysate, Initiation Factors, Ribosomes, and [^{35}S]Met-tRNA$_f^{Met}$. Preparation of rabbit reticulocytes, lysate, salt-washed ribosomes, and crude initiation factors was as described earlier (Gilbert and Anderson, 1970; Kappen et al., 1973; Kappen and Goldberg, 1973). Rabbit liver tRNA (General Biochemicals) was charged with [^{35}S]methionine (160 Ci/mmol, New England Nuclear) with *Escherichia coli* synthetase as described (Gupta et al., 1970; Kappen et al., 1973). Met-tRNA$_f^{Met}$ binding factors were prepared as reported by Suzuki and Goldberg (1974), and were a gift from Dr. Suzuki.

Binding of [^{35}S]Met-tRNA$_f^{Met}$ to Ribosomes and Formation of Peptides. Incubation conditions for the binding of [^{35}S]Met-tRNA$_f^{Met}$ in lysate were as described earlier (Kappen and Goldberg, 1973). The reaction mixture in a final volume of 0.1 ml contained 10 mM Tris-HCl (pH 7.5), 100 mM KCl or ammonium acetate, 2 mM $MgCl_2$, 0.4 mM GTP, 1 mM ATP, 0.04 mM each of 20 amino acids, 3 mM 2-mercaptoethanol, 15 mM creatine phosphate, 1 IU of creatine phosphokinase, 40 μl of 1:1 lysate, 30 μM hemin, and [^{35}S]Met-tRNA$_f^{Met}$ at specific activities indicated in the figure legends. At the end of incubation the reaction mixture was diluted fourfold with buffer containing 100 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM potassium cacodylate (pH 5.5). Aliquots were analyzed on 15–30% linear sucrose gradients, made up in the dilution buffer. The Mg^{2+} in the sucrose solution was always adjusted to the same level as in the incubation. After centrifugation in a Spinco SW41 rotor at 2 °C the gradients were fractionated as described earlier (Kappen et al., 1973). The fractions were counted in Bray's solution.

Reaction conditions for the binding of [^{35}S]Met-tRNA$_f^{Met}$ in the fractionated system were the same as described above for the lysate except that 0.5 M KCl washed ribosomes and crude initiation factors (KCl wash) were substituted for lysate.

Isolation of Ribosomes for Peptide Analysis. To determine the nature of the ribosome-bound [^{35}S]methionine, the ribosomes were isolated from incubations described above. The reaction mixture (0.2 ml) was diluted threefold with buffer and 500-μl aliquots were layered onto 7 ml of 15% sucrose solution containing 100 mM KCl, 1 mM dithiothreitol, 0.1 mM potassium cacodylate, and $MgCl_2$ at the same concentrations as in the incubations. After centrifugation at 48 000 rpm for 2 h at 2 °C in a 50 Ti rotor, the ribosomal pellet was suspended in buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol. In all cases aliquots of the ribosomal suspension were checked for absorbance at 260 nm and radioactivity. Recovery of the ribosomes from identical reaction mixtures was always within 5%. Ribosome-bound peptides were released from tRNA by incubation with 40 mM NaOH for 15 min at 37 °C and were analyzed by paper electrophoresis.

Incubation of Intact Reticulocytes. Reticulocytes were washed twice with a solution containing 0.14 M NaCl, 1.5 mM $MgCl_2$, and 5 mM KCl (special saline) and incubated under conditions similar to those described by Colombo et al. (1966). In addition, 1 ml of incubation solution contained 25 μCi of [^{35}S]methionine (160 Ci/mmol, New England Nuclear) and 0.5 ml of cell suspension. After 5-min incubation at 37 °C, the cells were washed twice with special saline and lysed with either 1.5 mM $MgCl_2$ or 1.5 mM $MgCl_2$ containing 0.1 mM aurintricarboxylic acid (ATA).[1] The suspension was centrifuged at 15 000g for 15 min. Aliquots of the lysate were analyzed for hot trichloroacetic acid insoluble radioactivity. The rest of the lysate was centrifuged through a cushion of 30% sucrose at 48 000 rpm (50 Ti rotor) for 2 h at 2 °C. The ribosomal pellet was hydrolyzed with alkali to liberate tRNA-linked peptides and the latter were analyzed by paper electrophoresis.

Electrophoresis. Procedures for electrophoresis were essentially the same as described (Kappen and Goldberg, 1973). The samples were applied on Whatman 3MM paper and subjected to electrophoresis at pH 8.0 in a system containing barbital (0.01 M), sodium barbital (0.015 M), triethylamine (0.03 M), and acetic acid in a Savant flat plate electrophorator. The paper was dried and stained with ninhydrin (0.3%) in acetone containing 10% acetic acid to locate unlabeled marker oligopeptides. One-centimeter strips were cut and the radioactivity was determined in Liquiflor toluene at an efficiency of 55% for ^{35}S.

Tritiated Pactamycin. Pactamycin was catalytically exchanged with tritium (performed at the New England Nuclear Corporation) and purified by two-dimensional thin-layer chromatography on an 8 in. × 8 in. 1000 μm silica gel G plate (Analtech). Solvent systems were: first, 2-propanol–methanol (75:25, v/v) (R_f 0.9); second, 1-butanol–formic acid (84:16, v/v) (R_f 0.7). Radioactive PM was detected by its fluorescence with ultraviolet light, eluted with methanol, and stored at −20 °C. The labeled PM was diluted with unlabeled PM to the required specific activity. The conditions used for the binding of [^{3}H]PM to the ribosomes are described in the legends to the figures.

Results

We have previously shown that about 50% of the ribosome-bound [^{35}S]methionine isolated from reticulocyte lysate incubated with [^{35}S]Met-tRNA$_f^{Met}$ in the presence of PM is in Met-Val with little in higher peptides, suggesting a block after the first peptide is formed (Kappen and Goldberg, 1973). In order to ascertain to what extent PM exerts its action by a block after Met-Val rather than at an earlier step (i.e., "joining") one needs quantitative data on the effect of the drug in a system that permits initiation and peptide formation at a certain number of initiation sites on the mRNA, but prevents chain elongation and termination. We therefore studied the effect of PM in lysates incubated with [^{35}S]Met-tRNA$_f^{Met}$ under standard conditions of protein synthesis in the presence of the antibiotic sparsomycin, which allows initiation and formation of short oligopeptides (Kappen and Goldberg, 1973; Smith and Wigle, 1973) but inhibits extended peptide chain elongation (Goldberg and Mitsugi, 1967). The ribosome-bound radioactivity was analyzed and the labeled peptides were quantitated. The results are summarized in Table I. With sparsomycin alone

Table I: Effect of PM on Peptide Synthesis in Reticulocyte Lysate.[a]

Expt.	Mg^{2+} (mM)	Radioact. in Peptides: Sparsomycin	Radioact. in Peptides: Sparsomycin + PM	% Peptides Inhibited by PM
1	2	9 752	3720	62
	5	6 920	4609	33
2	1.5	10 652	5392	50
	3	8 443	5652	33
3	1.5	9 277	7340	21
	3	9 780	7197	26

[a] Ribosomes isolated from 200-μl incubations (5 min at 30 °C) containing lysate and [^{35}S]Met-tRNA$_f^{Met}$ (0.15 A_{260} unit, 3×10^5 cpm) were suspended in 75 μl of buffer as described under Materials and Methods. The recovery of the ribosomes was quantitative in identical reaction mixtures although the specific activity (cpm per A_{260} unit) was lowered by higher Mg^{2+}. The suspension (50 μl) was hydrolyzed; peptide markers were added and analyzed by paper electrophoresis. The total radioactivity associated with Met-Val and higher oligopeptides was determined. Sparsomycin (1×10^{-5} M) and PM (2×10^{-6} M) were present from the beginning of the reaction.

Table II: Effect of PM on the Binding of [^{35}S]Met-tRNA$_f^{Met}$ to Ribosomes in the Presence and Absence of Extra Met-tRNA$_f$ Binding Factors.[a]

	[^{35}S]Met (cpm)		
	40S	80S	Polysomes
− Factors			
Sparsomycin	13 606	9 599	11 526
Sparsomycin + PM	11 681	3 560	12 177
+ Factors			
Sparsomycin	13 244	12 597	13 038
Sparsomycin + PM	18 119	5 948	11 800

[a] Incubations (100 μl) (3 min at 30 °C) contained lysate, [^{35}S]-Met-tRNA$_f^{Met}$ (0.05 A_{260} unit, 2×10^5 cpm), and other components given in Materials and Methods. Sparsomycin (1×10^{-5} M) and PM (2×10^{-6} M) were present from the start of the reaction. To one set of identical reaction mixtures Met-tRNA$_f$ binding factors (10 μg of 0.16 M KCl fraction and 20 μg of 0.19 M KCl fraction) were added. At the end of the incubation the reaction mixture was diluted fourfold with buffer and 300-μl aliquots were analyzed on 15–30% linear sucrose gradients. The [^{35}S]Met bound to 40S, 80S, and polysome regions were quantitated.

[^{35}S]Met radioactivity was distributed in oligopeptides including Met-Val, and when PM was included essentially only the dipeptide was synthesized, as previously described (Kappen and Goldberg, 1973). At the concentrations of magnesium (1.5 or 2.0 mM) optimal for polypeptide synthesis PM does not cause any significant reduction in the total ribosome-bound radioactivity (includes methionine not in peptide) but inhibits peptides labeled in the presence of sparsomycin significantly, the extent of inhibition varying with different batches of lysates. In experiment 1, PM inhibits the peptides 62% as compared to 21% with a different batch of lysate used in experiment 3. In a fractionated system composed of salt-washed ribosomes and crude initiation factors, PM inhibition of 80S initiation complex formation and methionylpuromycin synthesis decreased with increasing Mg^{2+} concentrations (Kappen et al., 1973). In the lysate system, raising the Mg^{2+} concentration to 3 or 5 mM, levels that inhibit Met-tRNA$_f^{Met}$ binding and polypeptide synthesis (L. S. Kappen and I. H. Goldberg, unpublished results), usually partially relieves PM inhibition of peptide synthesis (Table I). This was not seen in experiment 3, however, where the inhibition found at the low Mg^{2+} concentration was very low to start with. Such a result would be expected if this lysate contained sufficient "joining factor" to permit almost complete joining on a single cycle of use.

Inhibition of peptides by PM without significant reduction in the total ribosome-bound Met-tRNA$_f^{Met}$ implies that part of the initiation complex formed in the presence of PM is functionally inactive and this result could be accounted for by a defect in the association of the 60S ribosomal subunit with the initiation complex on the 40S subunit to form a complete initiation complex. Sucrose gradient analysis of incubation mixtures identical with those used for peptide analysis confirms this. As shown in Table II addition of PM significantly inhibits 80S complex formation. The degree of inhibition of the 80S complex ranged from 50 to 70% with different batches of lysates. It should be noted that unlike in the fractionated system the block in formation of the 80S complex is not always accompanied by an increase in 48S complex. The failure to obtain in all lysates quantitative accumulation of 48S complex following the inhibition of 80S complex formation suggests that the 48S complex formed in the presence of PM may be more susceptible to degradation, since PM does not inhibit its formation as such. Earlier observations in our laboratory (H. Suzuki and I. H. Goldberg, unpublished results), showing that the isolated 48S complex requires for its stability factors that mediate its formation, suggested that addition of such factors to the incubation might stabilize the 48S complex under conditions that lead to its accumulation. As predicted, addition to the lysate system of extra Met-tRNA$_f^{Met}$ binding factors (Suzuki and Goldberg, 1974) causes most of the radioactivity lost in the 80S complex to appear as the 48S complex (Table II). The two initiation factors that elute from a DEAE-cellulose column at KCl concentrations between 0.14–0.16 and 0.16–0.19 M are required for the formation of the tertiary complex and the smaller initiation complex, but are devoid of joining activity. The amount of [^{35}S]Met bound to the polysomes, although accounting for a large proportion of the total radioactivity bound to ribosomes, is not significantly affected by addition of PM, but that much of it is unable to participate in formation of the first peptide bond is evident from peptide analysis data (Table I).

That the functional defect in the polysome-bound complex is also due to lack of joining of the 60S subunit to the 48S complex at the initiation site on the messenger RNA is clearly indicated on sucrose gradients by the presence of radioactive peaks corresponding to "half-mer" oligoribosomal structures (Figure 1). In the absence of PM, the radioactive peaks coincide with the absorbance peaks, corresponding to mono, di-, and trisomes. Upon addition of PM additional radioactive peaks appear in between these peaks. It should be pointed out that the half-mer peaks are formed whether or not the extra Met-tRNA$_f^{Met}$ binding factors are added.

The most convincing evidence for the presence of half-mers in the polysome region comes from experiments with pancreatic ribonuclease. Incubations of lysate with [^{35}S]Met-tRNA$_f^{Met}$ in the presence of added factors were treated with RNase at 0 °C before analysis on sucrose gradients. Identical incubations containing no additional initiation factors were performed and analyzed. In the control containing only sparsomycin, RNase cleavage of the polysomes results in a quantitative shift of radioactivity from

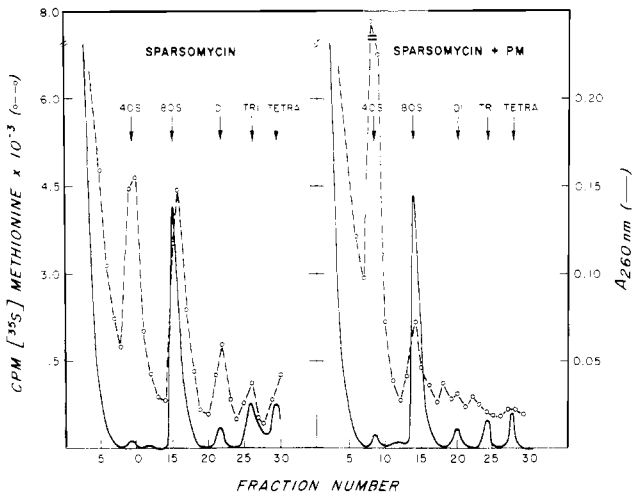

FIGURE 1: Effect of PM on the binding of [^{35}S]Met-tRNA$_f^{Met}$ to ribosomes in the presence of sparsomycin. Experimental conditions are the same as in Table II (+ Factors). Aliquots (300 μl) were analyzed on 15–30% linear sucrose gradients. Centrifugation was for 2 h at 41 000 rpm in an SW41 rotor. The positions on the gradient corresponding to the 40S subunit, monosome, disome, trisome, and tetrasome are indicated.

Table III: Effect of RNase Treatment of [^{35}S]Met-tRNA$_f^{Met}$ Bound to Polysomes in the Presence of PM.[a]

	[^{35}S]Met Bound (cpm)			
	48S	80S	Poly-somes	Total Ribosome Bound Radioact.
− Factors				
Sparsomycin	9 281	5 480	6346	21 107
Sparsomycin + PM	14 344	3 000	6280	23 624
Sparsomycin + RNase	10 740	9 872	2220	22 832
Sparsomycin + PM + RNase	11 846	4 131	1120	17 097
+ Factors				
Sparsomycin	9 248	9 573	6562	25 383
Sparsomycin + PM	13 018	4 581	5437	23 036
Sparsomycin + RNase	8 861	13 642	3246	25 749
Sparsomycin + PM + RNase	16 019	5 121	2521	23 661

[a] Effect of RNase treatment on [^{35}S]Met-tRNA$_f^{Met}$ bound to polysomes in the presence of PM. Four 100-μl incubations contained in addition to lysate, [^{35}S]Met-tRNA$_f^{Met}$ (0.15 A_{260} unit, 3×10^5 cpm) and other ingredients mentioned under Materials and Methods and Met-tRNA$_f^{Met}$ binding factors (40 μg of 0.16 M KCl fraction and 80 μg of 0.19 M KCl fraction). Sparsomycin (1×10^{-5} M) and PM (2×10^{-6} M) were present from the beginning of the reaction. After incubation for 3 min at 30 °C the tubes were chilled in ice. To one set of tubes was added pancreatic RNase to a final concentration of 1 μg/ml. After keeping on ice for 15 min, the reaction mixtures were diluted fourfold with buffer. Aliquots (250 μl) were analyzed on 12-ml linear sucrose gradients (15–30%). Centrifugation was for 110 min.

the polysomes to the 80S region of the gradient with little increase in the 48S complex (Table III; − Factors). When PM is present, however, only 20% of the radioactivity cleaved off from the polysome appears at the 80S region and the rest appears to have decomposed and gone to the top of the gradient. Part of the 48S complex in the control also has been degraded. On the other hand, in a similar incubation containing PM and additional Met-tRNA$_f^{Met}$ binding factors, RNase treatment again results in the shift of a small portion of the total radioactivity that disappeared from the polysome to the 80S ribosome, but the rest quantitatively goes to the 48S complex (Table III, + Factors). In the control with only sparsomycin, addition of factors does not alter the pattern of redistribution of radioactivity.

The foregoing results establish that in reticulocyte lysates PM can act on the joining of the 60S subunit to the 48S initiation complex probably under conditions where the joining factor is limiting and is prevented from recycling and, where joining has occurred, there still is a second block after the synthesis of the first peptide bond. The latter block can be shown to exist even at a high Mg^{2+} concentration where joining occurs to the same extent whether or not PM is present. Using crude initiation factors and salt-washed reticulocyte ribosomes bearing endogenous mRNA we have previously reported (Kappen et al., 1973; Goldberg et al., 1973) that the formation of a puromycin-reactive 80S initiation complex at 5 mM Mg^{2+} is not significantly affected by PM. To test whether this puromycin-sensitive initiation complex is functional or not in the subsequent steps of translation, the complex was further incubated in a complete amino acid incorporating system and the ribosome-bound radioactivity was analyzed to determine if it had been converted to peptides. As seen in Figure 2A in a control containing no PM, radioactivity is found in oligopeptides with a small amount in Met-Val. Analysis of the ribosome-bound radioactivity isolated from incubations containing 2×10^{-6} M PM shows that at least 50% of the total counts correspond to Met-Val, the initial peptide of globin chains, and most of the rest is in the methionine region (Figure 2B) with negligible amounts of other peptides. Evidence that the system permits the elongation of the peptide chains is provided by the results obtained with sparsomycin. As shown in Figure 2C, sparsomycin gives rise to di-, tri-, and higher oligopeptides. As expected, when both PM and sparsomycin are present oligopeptides are not formed (Figure 2D); all the radioactivity distributed in peptides in the sparsomycin treated sample shifts to Met-Val on addition of PM (Figures 2C and 2D).

Having found that a block after the first peptide bond is a common feature of PM inhibition in the cell-free system, partially fractionated or crude lysate, derived from rabbit reticulocytes, it was of interest to extend these studies to intact cells in which PM has been shown to inhibit protein synthesis and to lead to disaggregation of polyribosomes (Colombo et al., 1966). Analysis of ribosome-bound [^{35}S]Met isolated from reticulocytes incubated in the presence of PM is shown in Figure 3B. As in the cell-free systems radioactivity in the oligopeptides is almost entirely limited to Met-Val, although large amounts of radioactivity are also present at the origin and in Met. In the presence of PM radioactivity at the origin is due almost entirely to the internal labeling with methionine of preexisting chains undergoing completion. In the control (Figure 3A) where polysomes are maintained and contain peptides highly labeled with internal methionine, there is only a negligible amount of Met-Val. In order to rule out the possibility that Met-Val was formed after the lysis of the cells, lysis was carried out in the presence of ATA, an inhibitor of initiation (Grollman and Stewart, 1968) which blocks formation of the 80S complex and Met-Val synthesis (L. S. Kappen and I. H. Goldberg, unpublished results). The results obtained (Figure 3C) are identical with those in the absence of ATA (Figure 3B). These data confirm that PM also ex-

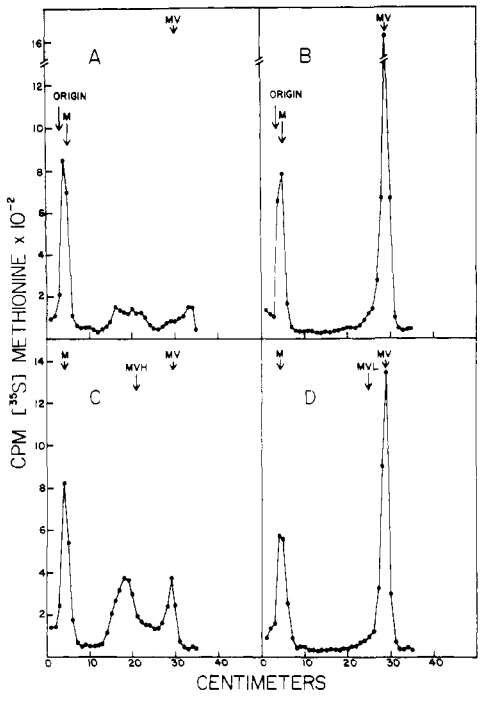

FIGURE 2: Electrophoretic analysis of ribosome-bound [^{35}S]Met isolated from an incubation containing salt-washed ribosomes and all the ingredients necessary for polypeptide synthesis. The incubation mixture (300 μl) contained 6.5 A_{260} units of 0.5 M KCl washed ribosomes, 800 μg of crude initiation factors, 0.5 A_{260} unit of [^{35}S]Met-tRNA$_f^{Met}$ (2×10^5 cpm), and other components listed under Materials and Methods. Incubation at 30 °C was done in two stages. During the first stage (10 min), the mixture contained all the components except amino acids, ATP, and an energy-generating system. At 10 min the latter components were added and the incubation was continued for an additional 7 min. PM and sparsomycin when present were added at the beginning. Analysis of the ribosome-bound radioactivity was done as described under Materials and Methods. Mg^{2+} in the reaction mixture and in the sucrose gradient was 5 mM: (A) control; (B) PM (2×10^{-6} M); (C) sparsomycin (1×10^{-5} M); (D) PM (2×10^{-6} M) and sparsomycin (1×10^{-5} M). M, MV, MVL, and MVH indicate the electrophoretic positions of marker methionine, Met-Val, Met-Val-Leu, and Met-Val-His, respectively.

erts a block after Met-Val in intact cells. On the other hand, cycloheximide, at concentrations (5×10^{-6} M) that inhibit protein synthesis by 90% in intact cells, leads to significant labeling of all oligopeptides including Met-Val (data not shown).

While it is clear from the foregoing that PM blocks the conversion of Met-Val to higher peptides when it is present from the start of the incubation, it was of interest to determine whether addition of PM after Met-Val formation would still interfere with this conversion. The experiment shown in Figure 4 was designed to answer this question. Lysate is incubated with [^{35}S]Met-tRNA$_f^{Met}$ in the presence of sparsomycin for 30 s by which time there is a predominant peak of radioactivity in Met-Val (Figure 4A). At 30 s, PM alone was added or PM followed by ATA to prevent further initiation complex formation, and incubation was continued to 2 min. As shown in Figure 4B, most of the Met-Val was converted to oligopeptides and the peptide distribution pattern is qualitatively similar to one obtained with sparsomycin alone (Figure 4D). In Figure 4C, ATA added immediately after PM prevents further binding and new Met-Val formation. In this case, although the total radioactivity is considerably decreased, most of the Met-Val formed prior to the addition of PM was converted to oligopeptides.

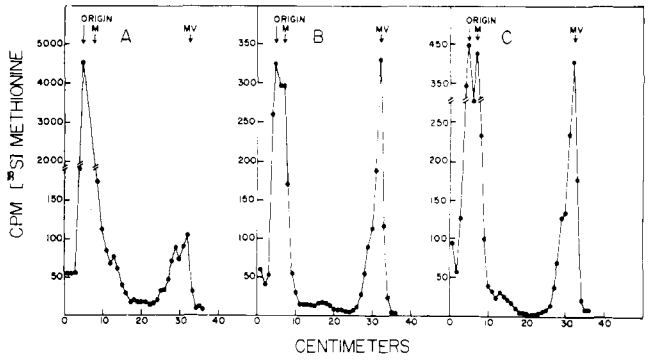

FIGURE 3: Analysis of ribosome-bound [^{35}S]Met isolated from intact reticulocytes. Ribosomes were isolated from reticulocytes labeled with [^{35}S]Met as given under Materials and Methods. In A and B cells were lysed with 1.5 mM $MgCl_2$ and in C with 1.5 mM $MgCl_2$ containing 0.1 mM ATA. The specific activities (counts per minute per A_{260} unit) are indicated in parentheses: (A) control (2893); (B) PM, 2×10^{-6} M (745); (C) PM, 2×10^{-6} M (714).

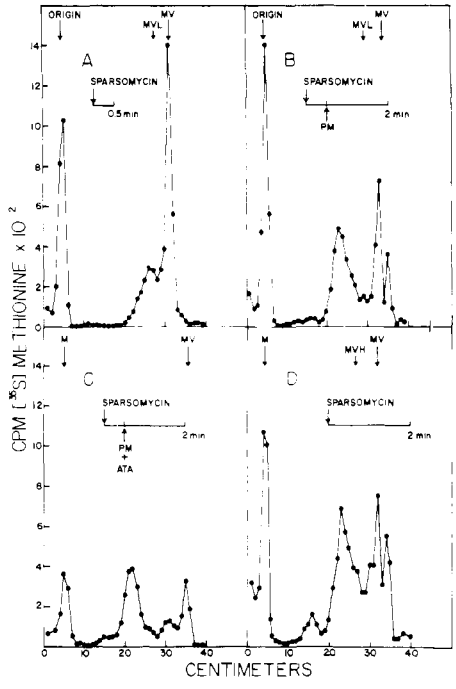

FIGURE 4: Effect of PM on the conversion of preformed Met-Val to oligopeptides. Four identical reaction mixtures (200 μl) contained lysate, all the components for protein synthesis, and [^{35}S]Met-tRNA$_f^{Met}$ (0.3 A_{260} unit, 2×10^5 cpm). Sparsomycin (1×10^{-5} M) was present from the beginning of the reaction. Other inhibitors were added at times indicated. Incubation was at 37 °C. Isolation of ribosomes and peptide analysis by electrophoresis were done as described under Materials and Methods. Except in A, incubation was for 2 min: (A) 30 s, control; (B) PM (2×10^{-6} M) was added at 30 s; (C) PM (2×10^{-6} M) and ATA (0.1 mM) at 30 s; (D) 2 min, control.

Additional evidence to support that PM interacts with the protein synthesizing complex only at or before the time of initiation, but not after chain elongation has begun, is provided by studies on [^{3}H]PM binding to ribosomes in lysate under conditions favoring initiation and not permitting polyribosomal degradation. Incubations included sparsomycin (1×10^{-5} M) which prevents the breakdown of polysomes but does not affect peptide chain initiation. The binding of [^{3}H]PM to polysomes in a reticulocyte lysate incubated with [^{35}S]Met-tRNA$_f^{Met}$ and other components necessary for protein synthesis is shown in Table IV. When

Table IV: [^{3}H]PM Binding under Initiating Conditions.[a]

Incubation Conditions	Time or Temp of Incubation	Radioact. Bound to the Polysomes (cpm) [^{35}S]Met	[^{3}H]PM
Experiment I			
– PM	8 min	11 539	
+ PM from 0 time	8 min	13 526	23 855
+ PM and ATA from 0 time	8 min	1 572	8 059
+ PM at 8 min	16 min	11 784	12 624
– PM	16 min	13 315	
Experiment II			
PM	0 °C	2 559	6 751
PM + ATA	0 °C	1 387	5 176
PM	30 °C	10 556	14 721
PM + ATA	30 °C	1 927	7 588

[a] Incubation mixture (200 μl) contains 80 μl of standard lysate, [^{35}S]Met-tRNA$_f^{Met}$ (2.5×10^6 cpm, 0.6 A_{260} unit) and other components indicated under Material and Methods. Sparsomycin (1×10^{-5} M) was present from the beginning of the reaction in all cases. PM (2×10^{-7} M, 3×10^5 cpm) and ATA (1×10^{-4} M) were added at times indicated. In experiment I incubation was at 30 °C and in II the reactions were incubated for 5 min at temperatures shown. After incubation, the reaction mixtures were diluted fourfold with buffer and 600-μl aliquots were analyzed on 15–30% linear sucrose gradients. The gradients were centrifuged at 41 000 cpm (SW 41 rotor) for 85 min and fractionated. The total radioactivity in fractions from the polysome regions (di- and higher) was determined.

Table V: Puromycin Reactivity of Methionylvaline Accumulated in the Presence of PM.[a]

	[^{35}S]Met-Val Bound to Ribosomes (cpm)	% Met-Val Released
10-min incubation	16 995	
17-min incubation	15 267	
ATA at 10 min, incubation continued for 17 min	16 161	
ATA + puromycin at 10 min, incubation continued for 17 min	3 668	79
Puromycin at 10 min, incubation continued for 17 min	3 693	78

[a] One milliliter of reaction mixture containing lysate, [^{35}S]Met-tRNA$_f^{Met}$ (0.5 A_{260} unit, 3×10^6 cpm) and all other components (Materials and Methods) was incubated in the presence of PM (2×10^{-6} M) for 10 min at 30 °C. The mixture was then distributed into five 150-μl aliquots, and one portion was chilled in ice. To the rest was added ATA (0.1 mM) and/or puromycin (1 mM) at times indicated, while the controls received an equal volume of H_2O. At the end of incubation (17 min) each sample was diluted with 200 μl of buffer. Aliquots (300 μl) from each were pelleted through a cushion of sucrose as described. The ribosomal pellet was suspended in 75 μl of buffer; 50 μl, after hydrolysis, was analyzed for peptides by paper electrophoresis.

[^{3}H]PM is present from the beginning of the reaction, there is a large increase of ^{3}H radioactivity in the polysomes concomitant with the binding of [^{35}S]Met. ATA inhibits the binding of both [^{35}S]Met-tRNA$_f^{Met}$ and [^{3}H]PM. By 8 min incubation most of the initiation complex has been formed and also most of the binding of [^{3}H]PM has taken place. There is a relatively high background of [^{3}H]PM on polysomes under conditions where initiation is inhibited by ATA or completed by preincubation or when initiation is minimal, e.g., at 0 °C (Table IV, experiment II). The significance of the binding found under noninitiating conditions is not clear, but may be due at least in part to the presence of preexisting sites at points of initiation. It is important to note, moreover, that the binding of [^{3}H]PM to polysomes is in any case much less than that to 80S ribosomes lacking mRNA or to free 40S subunits (Macdonald and Goldberg, 1970).

Whether the block after Met-Val formation is due to an effect on the first translocation was tested by addition of puromycin, an aminoacyl-tRNA analogue that releases peptides only from the ribosomal P-site. Addition of puromycin after the formation of the initial dipeptide in the presence of PM resulted in the release of almost 80% of the Met-Val (Table V). The same result was obtained when ATA was included to eliminate the possibility that new Met-Val might be formed and accumulate subsequent to the addition of puromycin. Similar experiments in the fractionated system showed that Met-Val accumulated at 5 mM Mg^{2+} in the presence of PM was 80% puromycin-releasable.

The accumulation of the initial dipeptide on ribosomes attempting polypeptide synthesis in the presence of PM implies that a qualitative difference exists between one of the steps involved in adding the second amino acid onto methionine and its counterpart concerned with adding subsequent amino acids. Such a possibility has been raised before (Kappen and Goldberg, 1973; Cheung et al., 1973) and is supported by experiments in which sparsomycin at levels 20 times that required to inhibit protein synthesis almost completely inhibits Met-Val accumulated in the presence of PM only slightly.

Discussion

PM inhibits methionylpuromycin synthesis and blocks 80S complex formation at 2mM Mg^{2+} in a system composed of salt-washed ribosomes and crude initiation factors derived from rabbit reticulocytes (Kappen et al., 1973). A similar effect of PM on the joining reaction has been reported in a different eucaryotic system by Levin et al. (1973). We have further shown (Kappen and Goldberg, 1973) in a globin-synthesizing reticulocyte lysate incubated with [^{35}S]Met-tRNA$_f^{Met}$ in the presence of PM that at least 50% of the ribosome-bound [^{35}S]Met is in Met-Val, the initial dipeptide of globin chains, indicating a block after the formation of the first peptide bond. Similarly, Met-Met has been found to accumulate in the presence of PM under conditions normally producing poly(Met) (Cheung et al., 1973). From our lysate experiments, however, it was not possible to ascertain the quantitative significance of the block after Met-Val since the effect of the drug was not compared with a "control" that permits only initiation and limited peptide bond formation. The experiments described here using sparsomycin provide conditions that permit one to compare the effect of a drug on initiation and peptide bond formation at a fixed number of initiation sites on the mRNA without chain release.

If PM exerts a block only after the first peptide is formed and not at an earlier step, e.g. initiation complex formation, one would expect that addition of the antibiotic would result in a quantitative shift of sparsomycin oligopeptides to Met-Val. Peptide analysis in several batches of lysates showed that addition of PM to a sparsomycin-containing incubation resulted in a loss of total methionine-containing peptide; the extent of inhibition of peptide varied with different lysates (Table I). This suggests that PM also acts be-

fore synthesis of the first peptide bond but after formation of the initiation complex on the 40S ribosomal subunit, since the total ribosome-bound Met radioactivity is not significantly affected by PM. We show that this block is at the joining of the 60S subunit to the 48S complex to form the 80S complex. The inhibition of the formation of the 80S complex in the lysate leads to an accumulation of the 48S complex in many but not all lysates, suggesting that the 48S complex may be destabilized in some lysates. We find that addition of 48S complex-forming factors confers stability to the complex and results in the quantitative accumulation of a 48S complex concomitant with the inhibition of the 80S complex. It is not clear, however, why extra factors are needed for its stabilization since in the fractionated system with crude or purified factors, PM does not inhibit the formation of the 48S complex (Kappen et al., 1973; H. Suzuki and I. H. Goldberg, unpublished results). It is possible that in these latter experiments the binding factors were always present in excess. By contrast, the [^{35}S]Met bound to polysomal structures is not reduced to any significant extent by PM in the absence of extra factors despite the fact that a large amount of it is in the form of oligosomes bearing a 48S initiation complex in the initiation region of the mRNA.

The most convincing evidence for the formation of "halfmer" type oligosomal structures in the presence of PM has been obtained by their cleavage by RNase in the presence of added factors. While RNase treatment of a control containing only sparsomycin resulted in the quantitative shift of radioactivity from the polysome to the 80S region, most of the radioactivity released from labeled polysomes in the presence of PM appeared in the 48S complex, confirming that 48S complex bound to the initiation site of the mRNA in the polysome did not have a 60S subunit attached to it.

We have earlier shown that in the fractionated system PM inhibition of methionylpuromycin synthesis and 80S complex formation is overcome by higher Mg^{2+} concentrations (Kappen et al., 1973). The present studies show that the 80S complex so formed in the presence of PM is functional to the extent of Met-Val synthesis but not beyond. This, taken together with the data on the accumulation of Met-Val in intact reticulocytes, indicates that in the reticulocyte system, intact cell or cell free, even if the defect in joining is overcome there still is a second block after the formation of dipeptide.

The finding that the antibiotic added after the formation of Met-Val is ineffective in blocking its conversion to oligopeptides (Figure 4) is consistent with the earlier observation that PM binds to free 40S ribosomal subunits or run-off ribosomes but not to ribosomes engaged in protein synthesis (Macdonald and Goldberg, 1970; Stewart and Goldberg, 1973). The present studies on the binding of [^{3}H]PM in lysate further confirm that the antibiotic interacts with polysomal complex mainly or only by virtue of its attachment to the 40S subunit during the early stages of initiation.

The above data establish that the inhibition of globin chain initiation in reticulocyte lysate by PM is due to its action at two loci: (1) inhibition of the joining of the 60S subunit to the 48S initiation complex, probably an indirect effect perhaps due to prevention of release and subsequent reuse of the joining factor, and (2) a block after the formation of the first peptide bond under conditions where joining has occurred. In most lysates 50 to 65% of the overall block in polypeptide formation can be accounted for by PM acting on (1) and the remainder is due to its action on (2).

The precise mechanism by which PM prevents oligopeptide formation is not known. Since the dipeptidyl moiety is puromycin reactive, it must have been placed into the ribosomal P-site; thus, PM does not inhibit the first translocation. Dipeptide accumulation has also been found in the presence of mycotoxin inhibitors of polypeptide chain initiation but this differs from the situation with PM since in the former the dipeptide is puromycin resistant (Mizuno, 1975). Whether the block by PM is at the level of the formation of the second peptide bond itself or results from interference with the second use of the acceptor site on the ribosome is not known. The fact that PM does not inhibit the puromycin release of nascent polypeptide (Cohen and Goldberg, 1967; Felicetti et al., 1966; Cundliffe and McQuillen, 1967) or dipeptide (this paper) suggests that peptide bond formation at any stage is not blocked. Since the puromycin reaction is only a model for natural peptide bond synthesis, this conclusion must, however, be tentative. In any case, a block after placement of the dipeptidyl-tRNA in the P-site requires that the second peptide bond formation or the second use of the acceptor site differs qualitatively from the first. There are no direct data on this point, although Met-Val formation appears to be relatively insensitive to sparsomycin which is highly effective in blocking methionylpuromycin, methionylvalylpuromycin, Met-Val-Leu, and Met-Val-His formation.

At this time in an effort to tie together the action of PM on the joining reaction with the block after Met-Val formation, we speculate that PM prevents the release of the joining factor from the initiation complex and thereby blocks a step involved in tripeptide formation, perhaps the one involving acceptor site function. Under conditions where joining depends on the catalytic reuse of the released joining factor, an overall block in 80S initiation formation is observed. This proposal bears certain similarities to the mechanism shown for the inhibition of acceptor site function and translocation by fusidic acid (Bodley et al., 1970; Cundliffe, 1972).

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Mechanism of Aminoacylation of tRNA. Proof of the Aminoacyl Adenylate Pathway for the Isoleucyl- and Tyrosyl-tRNA Synthetases from *Escherichia coli* K12†

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ABSTRACT: The following observations show that the formation of isoleucyl-tRNA catalyzed by the isoleucyl-tRNA synthetase from *Escherichia coli* K12 involves the initial rapid formation of an isoleucyl adenylate complex followed by the slow, rate-determining, transfer of the isoleucyl moiety to tRNA. (1) The rate constant for the transfer of [^{14}C]Ile from the E·[^{14}C]Ile~AMP complex to tRNA is the same as the turnover number for the steady-state isoleucylation of tRNA at pH 7.78 (1.5 s^{-1}) and pH 5.87 (0.34 s^{-1}). (2) On mixing a solution of isoleucyl-tRNA synthetase and tRNA with [^{14}C]Ile and ATP the steady-state rate of isoleucylation is attained in the first turnover of the enzyme, with little or no "burst" or charging that would indicate a slow step after the transfer step. (3) The pyrophosphate exchange reaction in the presence of tRNA is 40 times faster than the overall rate of isoleucylation of tRNA. (4) Similarly, rapid quenching experiments indicate that isoleucyl adenylate is formed prior to the transfer step. The possibility that isoleucyl adenylate formation is a parallel reaction caused by a second active site on the enzyme is ruled out both by the stoichiometry in this rapid quenching experiment and also the overall stoichiometry of isoleucyl-tRNA formation. At saturating reagent concentrations the major species in solution is the E·tRNA·Ile~AMP complex. Similar observations are found for the tyrosyl-tRNA synthetase except that at saturating reagent concentrations the rate constants for both tyrosyl adenylate formation and transfer are similar so that both processes contribute to the rate-determining step.

The mechanism of the aminoacylation of tRNA is a subject of some controversy. The classical mechanism, which involves the initial rapid formation of an aminoacyl adenylate complex followed by the transfer of the aminoacyl moiety to the tRNA, has been reviewed by Loftfield (1972) who argues in favor of a "concerted" mechanism in which tRNA, amino acid, and ATP react simultaneously with no intermediate being formed.

One of the arguments against the classical mechanism is that the rate constants for the transfer of the aminoacyl moiety from the E·AA~AMP complexes are too low to account for the observed turnover numbers for the aminoacylation of tRNA. However, these measurements have always been made under conditions that are far from optimal or those found in vivo.

A strategy for determining the mechanistic pathway is to measure: (a) the rate of transfer of the amino acid from the aminoacyl adenylate complex to tRNA to see if it is compatible with the stepwise mechanism; (b) the rate of formation of aminoacyl adenylate in the presence of tRNA, and the rate of aminoacylation of tRNA to see if the aminoacyl adenylate step is faster than the aminoacylation. There are two caveats. First, it is not sufficient in b to measure the steady-state rate of aminoacylation of tRNA since the rate-determining step in this process might be subsequent to the chemical aminoacylation step, for example, the rate-determining diffusion of the charged tRNA from the enzyme as has been found for the isoleucyl-tRNA synthetase from *Escherichia coli B* (Yarus and Berg, 1969; Eldred and Schimmel, 1972). Second, as it has now been demonstrated that even the monomeric aminoacyl-tRNA synthetases have at least two potentially active sites (Fersht, 1975), it must also be shown that the observed rates of aminoacyl adenylate formation and aminoacylation are not occurring separately and concurrently at two different sites.

We have made progress in this direction by introducing new techniques for measuring these rates and applying

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