PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES: EVIDENCE FOR THE SYNTHESIS OF INITIAL DIPEPTIDES IN THE PRESENCE OF PACTAMYCIN

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Summary

The effects of pactamycin on peptide chain initiation were studied using a reconstituted system comprised of reticulocyte ribosomes, poly r(A-U-G) messenger and peptide chain initiation factors, and the transfer of methionine from precharged Met-$tRNA_f^{Met}$ into di-, oligo- and polymethionine products was measured. In the presence of low concentrations of pactamycin (10^{-6}M), polymethionine synthesis in the above system was markedly reduced, and a concomitant increase in the synthesis of met-met and met-val dipeptides was observed. The latter dipeptide was presumably synthesized in response to endongenous hemoglobin messenger.

Aurintricarboxylic acid and sparsomycin also inhibited polymethionine synthesis in response to poly r(A-U-G) messenger. Whereas aurintricarboxylic acid inhibited pactamycin induced dipeptide synthesis, sparsomycin had no significant effect on dipeptide synthesis in the presence of pactamycin.

Pactamycin, at low concentrations (10^{-6}M), selectively inhibits peptide chain initiation in eukaryotic cells and also in cell-free extracts (1-6). Pactamycin binds to the 40 S subunit of ribosomes (1, 3-5) and causes an orderly dissociation of polyribosomes, which under appropriate conditions accumulate as monoribosomes (2-3). Pactamycin does not prevent the binding of Met-$tRNA_f^{Met}$ to reticulocyte ribosomes (5). In similar studies using *E. coli* ribosomes it has been shown that pactamycin does not prevent fMet-$tRNA_f^{Met}$ dependent binding of f2 viral RNA to *E. coli* ribosomes (7). Apparently, some of the accumulated monosomes are presumably in the form of inactive initiation complexes.

In order to determine the extent to which pactamycin-induced, defective initiation complexes are capable of synthesizing met-peptides, we studied

the effects of this inhibition on protein synthesis initiation using a reconstituted system comprised of reticulocyte ribosomes, poly r(A-U-G) messenger and partially purified peptide chain initiation factors (8-12). Pactamycin (10^{-6}M) in the above system inhibited the synthesis of poly- and oligomethionine products and caused an accumulation of the initial met-met dipeptide. The synthesis of the initial dipeptide of hemoglobin, met-val, during hemoglobin synthesis in reticulocytes and in the presence of inhibitory concentrations of pactamycin, has also been observed by Kappen and Goldberg (13).

MATERIALS AND METHODS

The preparations of preincubated reticulocyte ribosomes and ribosomal 0.5M KCl wash (I fraction) were the same as previously described (8-12). The crude I fraction was further purified by passage through a DEAE-cellulose column to remove Met-$tRNA_f^{Met}$ deacylase activity (11). The Met-$tRNA_f^{Met}$ deacylase activity eluted with the 0.1M KCl wash of the column and a partially purified mixture of the peptide chain initiation factors was obtained by further washing the column with 0.3M KCl (fraction II). This fraction II preparation was thoroughly dialyzed against Buffer D (5mM Tris-HCl, pH 7.5; 100mM potassium chloride; 1mM dithiothreitol; and 50 μM EDTA) and stored in ice.

Peptide chain initiation was assayed using poly r(A-U-G) messenger as described previously (12). The transfer of methionine from [^{35}S]Met-$tRNA_f^{Met}$ into di-, oligo- and polypeptides was measured using a paper chromatographic procedure.

Samples of pactamycin and sparsomycin were kindly donated by Dr. Alfred R. Stanley, National Institute of Health and G. S. Fonken, Upjohn Chemical Company, Kalamazoo, Michigan.

Other materials and methods were the same as previously described (8-12).

RESULTS

Initial Dipeptide Synthesis in the Presence of Pactamycin: We

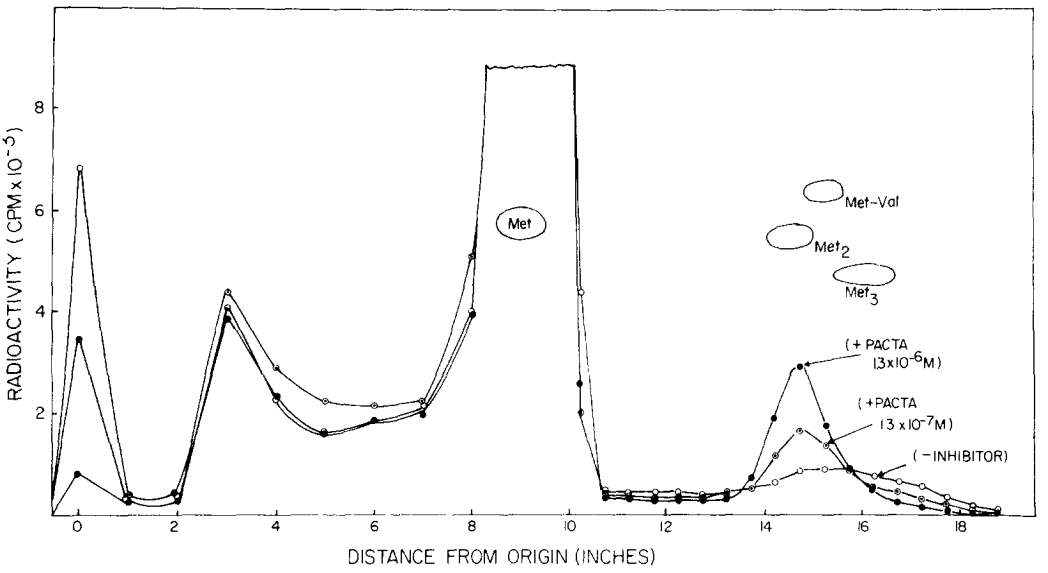

Figure 1. Paper chromatographic assay for di-, oligo- and polymethionine products synthesized in response to poly r(A-U-G) messenger and in the presence and absence of pactamycin. Poly r(A-U-G) directed [^{35}S]methionine transfer reaction was carried out as described previously (12). As before, a two stage procedure was used. In stage I, poly r(A-U-G) messenger was synthesized by transcription of the double stranded DNA-like polymer poly[d(A-T-C)]:poly[d(G-A-T)] using E. coli RNA polymerase and rATP, rUTP, and rGTP. The Stage II reaction mixtures (total volume 0.075 ml) contained usual protein synthesizing components (12) including 1 A_{260} unit washed preincubated reticulocyte ribosomes, 5 pmoles [^{35}S]Met-tRNA$_f^{Met}$ (10^5 cpm) present in approximately 0.2 A_{260} unit crude reticulocyte tRNA, 15 µg partially purified peptide chain initiation factors (fraction II) and where indicated pactamycin at the concentrations shown in the parenthesis. The reaction mixtures minus ribosomes were incubated in ice for 15 minutes and the reactions were started by addition of ribosomes. The reaction mixtures were then incubated at 37° for fifteen minutes. The reactions were then terminated by adding 5 µl 1.5N NaOH and the solutions were further incubated for one hour at 37° C. A 60 µl aliquot of the reaction mixture was then applied to paper chromatogram (Whatman 1M) beside marker amino acids and peptides. The chromatogram was developed using a solvent system composed of butanol:acetic acid:water (100:30:25). The chromatogram was then cut into one or one-half inch pieces and counted for radioactivity in toluene containing 4g per liter of Omnifluor (New England Nuclear).

studied the effects of pactamycin on peptide chain initiation using a reconstituted system comprised of washed and preincubated reticulocyte ribosomes, poly r(A-U-G) messenger and peptide chain initiation factors and measured the transfer of methionine from [^{35}S]Met-tRNA$_f^{Met}$ into di-,

oligo-, and poly-methionine products by paper chromatography. The results of a typical paper chromatographic assay method, in the presence and absence of pactamycin is shown in Fig. 1.

The radioactivity at the origin of the paper chromatogram represents newly synthesized polymethionine products. This synthesis of polymethionine is dependent on poly r(A-U-G) messenger and peptide chain initiation factors. In the absence of poly r(A-U-G) messenger the radioactivity at the origin was approximately 10-15 percent of that observed in the presence of poly r(A-U-G) messenger and was probably due to the synthesis of hemoglobin in response to remaining endogenous hemoglobin messenger. The second radioactive peak corresponded to a mixture of methionine sulfone and sulfoxide, and was observed in all the experiments. The third peak represents unreacted methionine. The radioactivity in the area beyond the methionine peak represents mostly di- and tripeptides of methionine. The authentic markers met-met, met-val and met-met-met moved in the region as indicated. The tripeptide met-met-met moved ahead of met-met and met-val dipeptides, and met-val dipeptide was distinctly ahead of met-met dipeptide. The synthesis of these short oligopeptides was also dependent on the addition of poly r(A-U-G) messenger and peptide chain initiation factors. In the presence of pactamycin, polypeptide synthesis (radioactivity at the origin) was markedly reduced. Approximately 50 and 90 percent inhibition of polypeptide synthesis was observed in the presence of 1.3×10^{-7} M and 1.3×10^{-6} M pactamycin respectively. Concomitant with the inhibition of polypeptide synthesis in the presence of pactamycin, an accumulation of a radioactive peak corresponding to the met-met region was observed. Also, in the presence of pactamycin, the radioactivity at the adjacent short peptide regions, probably due to met-met-met, is considerably reduced. These results would therefore suggest that in the presence of inhibitory concentrations of pactamycin, only the initial dipeptide, met-met, in response to poly r(A-U-G) messenger, is synthesized and the synthesis of tri- and longer peptides is inhibited.

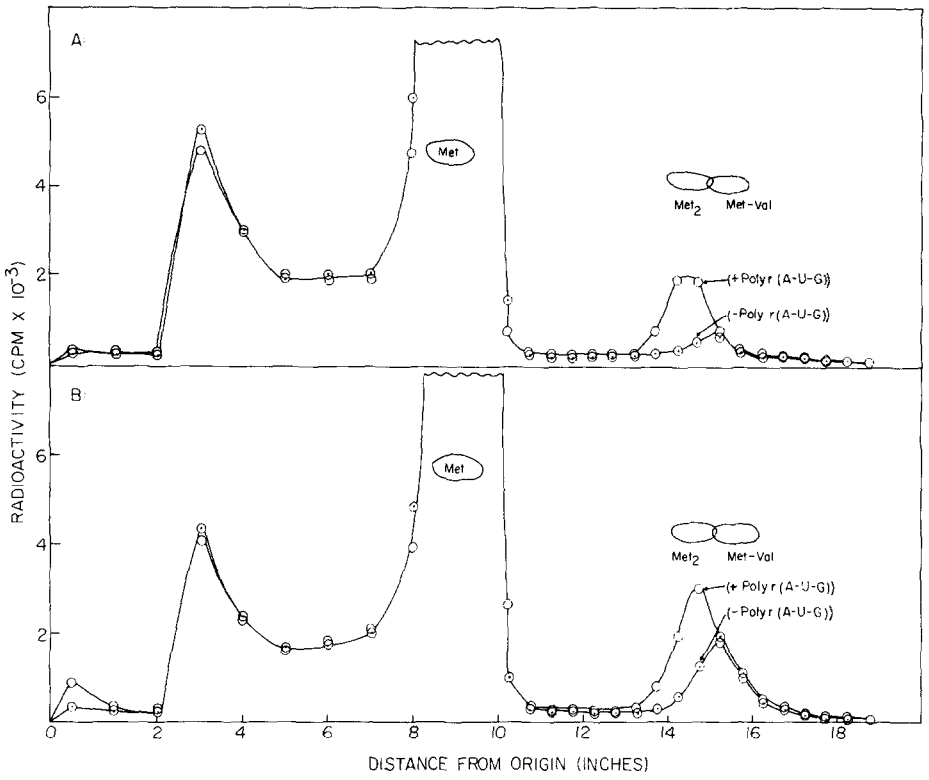

Figure 2. Effects of ribosome concentrations on the nature of pactamycin induced dipeptide synthesis in the presence and absence of poly r(A-U-G) messenger. The experimental procedures were the same as described in Fig. 1 except the experiments described in Fig. A contain 0.4 A_{260} unit ribosomes per 0.075 ml reaction mixture and the experiments described in Fig. B contain 2 A_{260} unit ribosomes. Poly r(A-U-G) messenger was omitted in the experiments described in curve ⊙-⊙-⊙.

The results presented in Fig. 2 show the effect of increasing ribosome concentrations on the nature of dipeptide synthesis in the presence of pactamycin. At a low ribosome concentration (0.4 A_{260} unit per 0.075 ml reaction mixture) (Fig. 2A) the dipeptide synthesis was clearly dependent on the addition of poly r(A-U-G) messenger, and the dipeptide thus synthesized moved precisely with the met-met marker. In the absence of poly r(A-U-G), a small radioactive peak corresponding to met-val dipeptide and distinct from met-met marker, was observed. At higher ribosome concentration (2 A_{260} unit per 0.075 ml reaction mixture) (Fig. 2B), and in

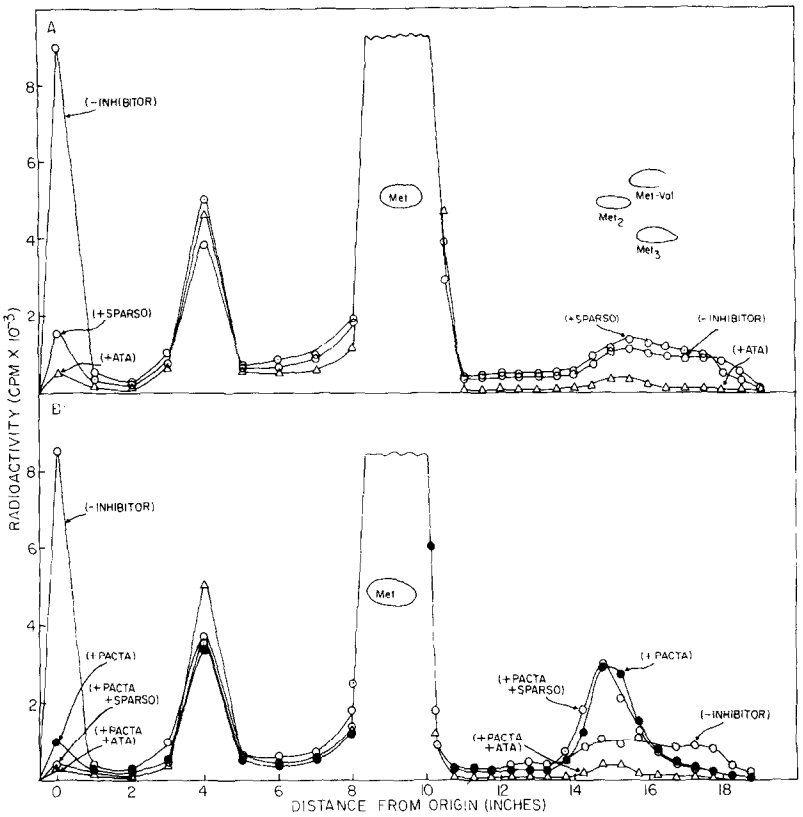

Figure 3. Effects of aurintricarboxylic acid and sparsomycin on di-, oligo-, and polypeptide synthesis in the presence and absence of pactamycin. The experimental procedures are the same as described in Fig. 1 except that aurinticarboxylic acid (1×10^{-4} M), sparsomycin (1.3×10^{-5} M) and pactamycin (1.3×10^{-6} M) were added singly or in combinations, to the reaction mixtures as shown in the graph.

the presence of poly r(A-U-G) messenger, the radioactivity peak at the di-peptide region is somewhat broader and is presumably due to over-lapping of met-met and met-val dipeptides. In the absence of poly r(A-U-G) messenger, the radioactivity peak at the dipeptide region was now shifted towards right and is coincident with met-val marker.

Effects of Aurintricarboxylic Acid (ATA) and Sparsomycin: ATA inhibits peptide chain initiation in reticulocytes presumably by preventing the formation of the initiation complex Met-$tRNA_f^{Met}$:IF1:GTP (12). Sparsomycin is known to inhibit the peptidyl synthetase reaction (14-15).

Both these inhibitors, at low concentrations, also inhibited polypeptide synthesis (Fig. 3A, the radioactivity at the origin of the chromatogram).

In the presence of ATA, there was also a sharp reduction of radioactivity at the short peptide region. This result is consistent with the interpretation that ATA prevents the formation of the initial initiation complex Met-$tRNA_f^{Met}$:IF1:GTP (12). In the presence of inhibitory concentrations of sparsomycin, there was little change in the radioactivity at the short peptide region although polypeptide synthesis was sharply reduced.

The results shown in Fig. 3B show the effects of ATA and sparsomycin on pactamycin induced dipeptide synthesis. Again as expected, ATA drastically inhibited pactamycin induced dipeptide synthesis and the total radioactivity at the short peptide region in the presence of both ATA and pactamycin was very low. In the presence of inhibitory concentrations of sparsomycin, however, there was almost no change in the extent of pactamycin induced dipeptide synthesis. These experiments were done at several levels of sparsomycin (10^{-6} - 10^{-5} M) and the results were similar; polypeptide synthesis was inhibited but no significant change in pactamycin induced dipeptide synthesis was observed. Also, hemoglobin messenger directed met-val dipeptide synthesis in the presence of pactamycin was unaffected by sparsomycin.

DISCUSSION

The data presented in this paper clearly demonstrate that in the presence of inhibitory concentration of pactamycin, the initial dipeptides, met-met corresponding to poly r(A-U-G) messenger and met-val corresponding to hemoglobin messenger, are synthesized. These results together with previous observations (3-5, 7) suggest that pactamycin interacts with ribosomes and forms inactive initiation complexes in which only the initial dipeptide corresponding to the first two codons are synthesized, and further movement of the protein synthesizing machinery into

translocation along the messenger RNA is prevented. Sparsomycin, which strongly inhibited polypeptide synthesis presumably by inhibiting peptidyl synthetase (14-15), did not inhibit pactamycin induced dipeptide synthesis. In agreement with previous workers (16), we have observed that sparsomycin inhibits Met-puromycin synthesis, a model reaction widely used for studies of the synthesis of the initial dipeptides. Apparently, the characteristics of pactamycin induced initial dipeptide synthesis are different from those of Met-puromycin synthesis. Recently, Seal and Marcus (4) and also Kappen, Suzuki and Goldberg (5) reported that pactamycin inhibited Met-puromycin synthesis only if this antibiotic was added at the beginning of Met-$tRNA_f^{Met}$ binding to ribosomes, and that no inhibition of Met-puromycin synthesis was observed if pactamycin was added after Met-$tRNA_f^{Met}$ was preincubated with ribsomes for sometime. These authors suggested a two site mechanism for binding of Met-$tRNA_f^{Met}$ to ribosomes. In this mechanism Met-$tRNA_f^{Met}$ initially binds to site 1 and then moves to site 2 and becomes reactive to puromycin. This reaction of Met-$tRNA_f^{Met}$ in site 2 and puromycin is sensitive to sparsomycin action. Pactamycin presumably "freezes" Met-$tRNA_f^{Met}$ in site 1 and Met-$tRNA_f^{Met}$ in this site cannot react with puromycin. The results presented in this paper suggest that Met-$tRNA_f^{Met}$ in the presence of pactamycin, presumably in site 1, can interact with another aminoacyl tRNA bound to ribosomes in response to an adjacent codon and can synthesize the initial dipeptide. This interaction between Met-$tRNA_f^{Met}$ in site 1 and an adjacent aminoacyl tRNA is not sensitive to sparsomycin action. The characteristics of this pactamycin induced dipeptide synthesis is not apparent. It is conceivable that peptidyl synthetase catalyzes this initial dipeptide synthesis but is protected from sparsomycin action by the specific ribosomal conformation at this site. It is not clear if the characteristics of this initial dipeptide synthesis reflect the true nature of the initiation process or is a specific case induced by pactamycin. Further work will be necessary to define precisely

the nature of the initial dipeptide synthesis in the presence and absence of pactamycin and its relationship to Met-puromycin synthesis.

In the present work, we have demonstrated that pactamycin induces dipeptide synthesis corresponding to poly r(A-U-G) messenger and also hemoglobin messenger. This observation, therefore, raises the possibility that pactamycin may be used as a tool for studies of cistron-specific protein synthesis. A specific dipeptide synthesized in the presence of pactamycin may be used as a measure of initiation of a specific cistron or for detection of specific initiation sites on a segment of messenger RNA. Indeed, as a specific inhibitor of peptide chain initiation, pactamycin has recently been used to map the gene order of certain RNA viruses (17-18).

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