INHIBITION OF GLOBIN CHAIN INITIATION IN RETICULOCYTE LYSATES BY PACTAMYCIN: ACCUMULATION OF METHIONYL-VALINE

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SUMMARY

In a globin synthesizing system containing reticulocyte lysate and [^{35}S] met-tRNA$_f$, low levels of pactamycin cause an accumulation of radioactivity on the monosomes and small oligosomes concomitant with the breakdown of polysomes. About 50% of the ribosome-bound radioactivity corresponds to methionyl-valine, the initial dipeptide of globin chains, with negligible amounts of tri- or other oligopeptides. This suggests that the site of its action is after the formation of the first peptide bond. The ribosome-bound radioactivity in the presence of sparsomycin, an inhibitor of chain elongation, is in di-, tri- and oligopeptides. Sparsomycin levels (10^{-5}M) that cause almost complete inhibition of polypeptide synthesis have only a small inhibitory effect on the pactamycin-induced accumulation of methionyl-valine. Fusidic acid and chlortetracycline do not cause accumulation of any significant amounts of methionyl-valine.

Low concentrations of pactamycin (PM) specifically inhibit the initiation of polypeptide synthesis in intact cells and cell-free extracts of eucaryotes (1-3). It has been shown that PM binds to the smaller subunit of rabbit reticulocyte ribosomes and blocks the formation of new polypeptide chains while allowing the completion and release of globin chains previously started on polyribosomes (1,2). In reticulocyte lysates, PM induces a rapid and sequential breakdown of polyribosomes to monosomes while blocking the formation of new polyribosomes (2-4). Furthermore, globin synthesis stimulated by added initiation factors is more sensitive to inhibition by the antibiotic than synthesis that permits merely the completion of existing chains (5,6).

Pactamycin, as a selective inhibitor of polypeptide chain initiation, has been used as a tool in the study of biological processes (7-10); but the precise mechanism by which it interferes with initiation is not known.

The formation of a non-functional larger initiation complex in the presence of PM has been reported (11-13). In addition, while the factor- and GTP-dependent binding of [^{35}S] met-tRNA$_f$ to the 40S subunit of rabbit reticulocyte ribosomes is not affected by PM, the antibiotic prevents the formation of a stable, functional larger initiation complex (5,13). In this paper, we show that in a globin-synthesizing system containing rabbit reticulocyte lysate and [^{35}S] met-tRNA$_f$ in the presence of PM there is an accumulation of radioactivity on the mono- and small oligosomes following the breakdown of polyribosomes. About 50% of the ribosome-bound radioactivity is found in the form of methionyl-valine, the initial dipeptide of globin chains; and there is virtually no accumulation of the initial tripeptide or higher peptides, suggesting that the functional locus of PM action is after the formation of the first peptide bond. This result was not seen with antibiotic inhibitors of peptide chain elongation.

MATERIALS AND METHODS

Rabbit reticulocytes were prepared as described (2). The washed, packed cells were lysed with an equal volume of 2 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM EDTA and the suspension was clarified at 15000 x g for 20 minutes. The lysate was stored in liquid nitrogen in aliquots. Protein synthesis in lysate was measured by the incorporation of [^{14}C] leucine and [^{14}C] valine into hot trichloroacetic acid-precipitable material as reported(2).

Rabbit liver tRNA was acylated with [^{35}S] methionine (160 Ci/mmole) (New England Nuclear) using *E. coli* synthetase (13). The binding of [^{35}S] met-tRNA in lysate was carried out in incubations similar to those for protein synthesis and analysed on sucrose density gradients. A standard incubation contained per ml 0.03 mM hemin, 500 µl lysate, 100 mM ammonium acetate, 2 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5, 6 mM 2-mercaptoethanol, 1 mM ATP, 0.36 mM GTP, 0.055 mM cold amino acids, 15 mM

creatine phosphate, 100 µg creatine phosphokinase and [^{35}S] met-tRNA. Samples of this incubation were analysed on gradients under conditions given in the figure legends.

Samples for electrophoretic analysis were prepared from binding incubations described above. Fractions from the mono- and polysome region of the gradient were pooled, pelleted at 48000 rpm (50 Ti rotor) for 2 hours at 2°C. The pellet was suspended in 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol. The peptide was released by hydrolysis with 40 mM NaOH for 15 minutes at 37°C. Separation of peptide products by electrophoresis was performed by the procedure of Elson, Crystal and Anderson (14). The samples were spotted on Whatman 3 MM paper and subjected to electrophoresis at pH 8.0 (barbital (0.01 M), sodium barbital (0.015 M), triethylamine (0.03 M), acetic acid) in a Savant flat plate electrophorator. The paper was dried and stained with ninhydrin (0.3%) in acetone containing 10% acetic acid. One cm strips were cut and counted in Liquiflor toluene at an efficiency of 50% for ^{35}S.

Puromycin sensitivity of ribosome bound met-tRNA was tested in a second incubation using material isolated from the binding incubations on the gradient as described in electrophoresis. The incubations contained 100 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, ribosome suspension and 1 mM puromycin. The incubations were either at 37°C for 10 minutes or the mixture was kept in ice for 15 minutes. Aliquots were analysed on 15-30% linear sucrose gradients as described above.

RESULTS AND DISCUSSION

When reticulocyte lysate is incubated with [^{35}S] met-tRNA in the complete amino acid incorporating system containing hemin, a peak of radioactivity is associated with the 40S subunits (not shown) as well as with the polysomes (Fig. 1). A complex between the 40S subunit and met-$tRNA_f$ has been implicated as a natural intermediate in the process of initiation (15,16). The formation of such a complex is not inhibited

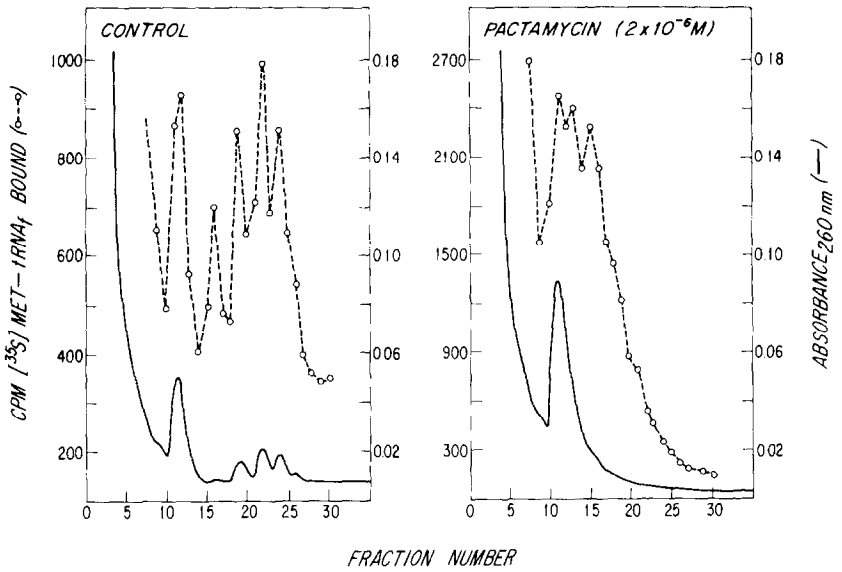

Figure 1. The labelling of polysomes with [^{35}S] met-tRNA and effect of pactamycin. Incubation mixture (0.2 ml) contained 0.24 OD_{260} units of [^{35}S] met-tRNA (cpm 150,000) and components for proteins synthesis at concentrations specified in the experimental section. In the panel on the right PM (2×10^{-6}M) was present from 0 time. Incubation was at 37°C. At 2 minutes, the mixture was diluted with 1.5 ml of ice cold buffer containing 25 mM KCl, 10 mM NaCl, 0.1 mM potassium cacodylate pH 5.5, 2 mM $MgCl_2$, 1 mM dithiotreitol and 1 ml was layered on 11 ml linear 15-30% sucrose gradients supported by a 1 ml cushion of 45% sucrose in 100 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol and 0.1 mM potassium cacodylate pH 5.5. Centrifugation was for 90 minutes at 41000 rpm in a Spinco SW41 rotor at 2°C. The gradients were fractionated and the fractions were counted for radioactivity as described (13).

by PM since during early stages of incubation the level of radioactivity bound at the 40S region is the same as in a parallel incubation lacking the antibiotic (data not shown). These results are similar to our earlier observations (5,13) with salt washed ribosomes that the factor- and GTP-dependent binding of [^{35}S] met-$tRNA_f$ to the 40S subunit is not affected by PM. However, in a 2 minute incubation PM causes the disaggregation of polysomes (2) with the accumulation of [^{35}S] radioactivity on the monosomes and the smaller oligosomes (Fig. 1). With time in the control the amount of [^{35}S] met associated with the ribosomes on the gradient decreases, presumably due to cleavage of the initial methionine from the elongating peptide chain. These findings indicate that in the presence of PM a 80S initiation complex is formed

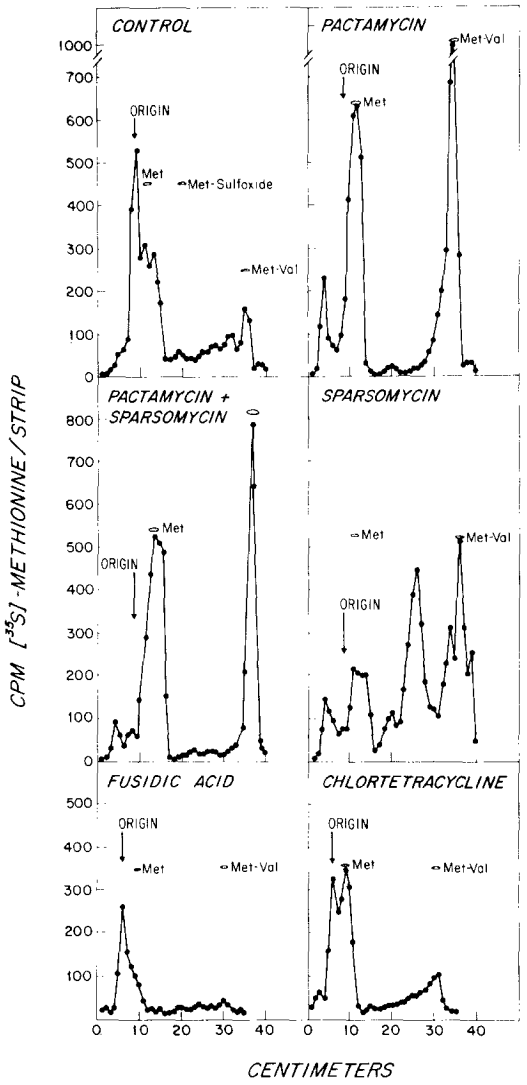

Figure 2. Electrophoretic analysis of the peptides synthesized in the presence of pactamycin and other inhibitors. 0.2 ml reaction mixtures were prepared, incubated and centrifuged through the gradient as described in Fig. 1. The inhibitors (PM, 2 x 10^{-6}M; sparsomycin, 1 x 10^{-5}M; fusidic acid, 2 x 10^{-3}M; chlortetracycline, 1 x 10^{-3}M) when present were added at the beginning. The polysome was isolated as described in methods. The pellet was suspended in 100 µl of buffer (see methods) and incubated with 4 µ moles of NaOH at 37°C for 15 minutes. Internal standards methionine (20 µg), methionine sulfoxide (20 µg) and methionyl-valine (40 µg) were added. 80 µl sample was applied on Whatman No. 3 MM paper. Counts applied per strip varied from 5,000-10,000. Electrophoresis was for 2.5 hr. at 3400 volts and 40 mA in the case of A,B,C,D and 2 hr. for E and F. The positions of the markers methionine (met), methionyl-valine (met-val) as located with ninhydrin are shown in the figure.

by the addition of a 60S particle to the 40S initiation complex, but this complex is defective in one of the subsequent steps of translation.

These results are in contrast to those in the fractionated system (5,13) where PM prevents the formation of a stable larger initiation complex with resultant accumulation of the smaller initiation complex. On the other hand, in the presence of PM a peak of radioactivity is found associated with particles (Fig. 1) that sediment more rapidly than monosomes but are slower moving than the disomes. Similar structures have been reported earlier (13).

In order to identify the nature of [^{35}S] radioactivity bound to the ribosomes, we isolated the mono- and oligosomes from sucrose density gradients and the bound radioactive product was liberated from tRNA and analysed by high voltage electrophoresis. Figure 2 shows the electrophoretic separation of the products of the ribosome-bound [^{35}S] radioactivity from incubations containing different antibiotics. In the presence of 2×10^{-6}M PM, about 50% of the total radioactivity per strip corresponded to methionyl-valine and most of the rest is in the region of methionine. Negligible radioactivity is in the tripeptide region. The percentage of met-val ranges from 45 to 52 in 15 experiments. As can be seen in Table 1, met-val accumulation is maximal at 2×10^{-6}M PM at which concentration polypeptide synthesis is 88% inhibited. The percentage of met-val decreases with decreasing concentrations of the antibiotic and is negligible at levels below 5×10^{-7}M PM. A control incubation containing no antibiotic shows only a small peak of met-val even at 0.5 minute incubation and at 2 minutes the predominant peak of radioactivity is at the origin associated with polypeptide. We compared the effects of three other antibiotics at concentrations that give comparable inhibition of globin synthesis in reticulocyte lysate. Sucrose density gradient analysis of reaction mixtures containing sparsomycin (1×10^{-5}M, 98% inhibition) shows that sparsomycin prevents the breakdown of polyribosomes while fusidic acid and chlortetracycline exert a similar but lesser effect. Polysome bound radioactivity isolated from

TABLE 1

Comparison of the effect of pactamycin and other inhibitors of protein synthesis on the accumulation of methionyl-valine*

Inhibitor	% inhibition of polypeptide synthesis	[^{35}S] methionine per strip	cpm [^{35}S] Met-Val	% Met-Val
None		4186	432	10.3
PM (2×10^{-7}M)	20	2629	575	21.8
(5×10^{-7}M)	59	4894	1959	40
(1×10^{-6}M)	77	4257	1831	43
(2×10^{-6}M)	88	4870	2269	46.5
(1×10^{-5}M)	95	4411	1832	41.5
SPAR (1×10^{-5}M)	98	6939	1275	18.3
FUS (2×10^{-3}M)	89	1522	126	8.2
CTC (1×10^{-3}M)	71	3068	421	13.7

*Incubation conditions and analysis by paper electrophoresis were identical to those in Fig. 2 except that polypeptide synthesis was determined by the incorporation of [^{14}C] valine and [^{14}C]leucine (2). SPAR = sparsomycin, FUS = fusidic acid, CTC = chlortetracycline.

sparsomycin-treated samples gave di, tri and other unidentified peaks presumably of higher oligopeptides (Fig. 2). Although not shown, the initial hemoglobin tripeptides were located with met-val-his and met-val-leu as markers. When both PM and sparsomycin are present during incubation, the latter only partially inhibits met-val synthesis. Fusidic acid at higher concentrations has been reported to inhibit met-val synthesis (17).

The puromycin reactivity of the [^{35}S] radioactivity bound to ribosomes in incubations containing 2×10^{-6}M PM was determined. We found that [^{35}S] met-tRNA$_f$ bound in the presence of PM is partially

(about 50%) releasable by puromycin on incubation at 37°C for 10 minutes, but the percent release was always less than in a control with no antibiotic. The reactivity to puromycin may be accounted for by a slow shift of the bound met-tRNA and met-val tRNA to the peptidyl site, although other explanations are also possible.

We have previously shown that PM produces a non-functional initiation complex with reticulocyte ribosomes bearing endogenous messenger (5,13). Similar results have been obtained in systems with wheat embryo ribosomes and TMV RNA as messenger (11,12). We now show that at least half of the 80S complex formed in the presence of PM in the more complex lysate system appears to be functional to the extent that the bound met-$tRNA_f$ is located on the "P" site and is capable of forming the first peptide bond. The accumulation of met-val, the initial dipeptide of globin chains, in the presence of PM suggests that the locus of its action is after the formation of the first peptide bond. These results are in accord with the findings of Cheung, Stewart and Gupta (accompanying paper in this volume) who observed an accumulation of dipeptide in the presence of PM in systems containing preincubated reticulocyte ribosomes and synthetic messenger. The accumulation of dipeptide in the presence of PM suggests that one of the next steps involved in the formation of tripeptide is inhibited. On the other hand, studies on polypeptide synthesis show that elongation of globin chains can normally proceed in the presence of these concentrations of PM. This can be explained if PM binds to the ribosome only at the time of initiation and not subsequently (5). The possibility exists, however, that one of the immediate steps following dipeptide formation is especially susceptible to PM, whereas the subsequent ones of chain elongation are relatively resistant. The fact that sparsomycin (10^{-5}M), an inhibitor of peptidyl transferase function (5), has only a small effect on met-val formation, as also found by Cheung, Stewart and Gupta (accompanying paper) in a different system,

also suggests the possibility that formation of the first peptide bond is in some way qualitatively different from the subsequent ones. It should be noted, however, that higher sparsomycin concentrations ($\geq 10^{-4}$M) show increasing inhibition of dipeptide formation.

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