Inhibition of Reticulocyte Peptide-Chain Initiation by Pactamycin: Accumulation of Inactive Ribosomal Initiation Complexes

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ABSTRACT Pactamycin does not inhibit the overall initiation factor- and GTP-dependent binding of [35S]Met-tRNA\textsubscript{f} to rabbit reticulocyte ribosomes but does prevent the formation of Met-puromycin, provided that the antibiotic is present during the course of the binding reaction. These data indicate that pactamycin blocks the synthesis of a functional peptidyl chain initiation complex. Sucrose density gradient centrifugation analysis of binding reactions shows that pactamycin causes the accumulation of an initiation complex on the smaller ribosomal subunit (smaller initiation complex), to which the 60S ribosomal subunit either cannot join or with which it forms a larger inactive 80S initiation complex that falls apart under the conditions used for isolation. The smaller initiation complex formed in the presence of pactamycin differs from the normal intermediate in peptide-chain initiation in being much more resistant to degradation by pancreatic RNase.

In the presence of pactamycin, the inactive smaller complex can also form on mRNA to which an unaffected ribosomal couple is already attached, forming an oligoribosome lacking a larger ribosomal subunit or a "1.5 mer." These effects of pactamycin can be overcome to a considerable degree by elevation of the Mg\textsuperscript{2+} concentration.

Low concentrations of the antibiotic pactamycin selectively inhibit the initiation of polypeptide synthesis in intact cells and ribosome-containing extracts of eukaryotes (1–8). Thus, we have shown that pactamycin binds to the smaller subunit of rabbit reticulocyte ribosomes and blocks the formation of new polypeptide chains, while allowing the completion of globin chains already started on polyribosomes (1, 2). In reticulocyte lysates, pactamycin permits the orderly decay of polyribosomes to single ribosomes, while blocking the formation of new polynucleosomal structures (2–4). Further, we have found that globin synthesis dependent on the addition of initiation factors to salt-washed ribosomes is sensitive to the antibiotic, whereas synthesis not requiring the starting of new polypeptide chains is resistant (5).

Although pactamycin, as an inhibitor of polypeptide chain initiation, has already proved to be a useful tool in determining the gene order of certain RNA viruses infecting animal cells (6–8), the precise mechanism by which the antibiotic interferes with initiation is not known. Several lines of evidence have suggested that the antibiotic produces an inactive initiation complex (2, 5, 9). In this paper we show that in the presence of pactamycin, the smaller initiation complex (40S ribosomal subunit-mRNA-Met-tRNA\textsubscript{f}) is formed at 2 mM Mg\textsuperscript{2+}, but it is unable to join with the 60S ribosomal subunit to form a functional, stable larger initiation complex. At higher Mg\textsuperscript{2+} concentrations, however, a functional larger initiation complex is formed despite the presence of pactamycin. The smaller initiation complex that accumulates with pactamycin at low Mg\textsuperscript{2+} concentrations is more resistant to pancreatic RNase than is the smaller initiation complex that is a normal intermediate in polypeptide chain initiation.

MATERIALS AND METHODS

Preparation of 0.5 M KCl-washed reticulocyte ribosomes and KCl wash (crude initiation factors) was as described by Gilbert and Anderson (10). Ribosomes were washed for only 10 min. The ribosomal salt-wash was treated with DEAE-cellulose, dialyzed, concentrated by the method of Gupta et al. (11), and stored in liquid nitrogen in small aliquots. Rabbit liver tRNA (General Biochemicals) was charged with [35S]-methionine (160 Ci/mmol) (New England Nuclear) with E. coli synthetase, which acylates only the formylatable species (12).

Met-tRNA\textsubscript{f} Binding Assay and Met-Puromycin Synthesis. The endogenous mRNA-dependent binding of Met-tRNA\textsubscript{f} was measured by Millipore filtration, essentially as described by Crystal and Anderson (13). For Met-puromycin synthesis, 0.5 mM (neutralized) puromycin dihydrochloride was added after binding had taken place, and incubation was continued for the time indicated in the legends at 30°. The reaction was terminated by the addition of 1 ml of 0.1 M potassium phosphate buffer (pH 8.0), and Met-puromycin was extracted with 3 ml of ethylacetate; 2 ml of the extract was counted in Bray's solution (14).

Sucrose Density Gradient Centrifugation of Met-tRNA\textsubscript{f} Bound to Ribosomes Bearing Endogenous mRNA. The binding of Met-tRNA to ribosomes was analyzed on 15–30% linear sucrose gradients containing 100 mM KCl-2 mM MgCl\textsubscript{2}-1 mM dithiothreitol-0.1 mM potassium cacodylate (pH 5.5) by the procedure of Crystal and Anderson (13), except that the Mg\textsuperscript{2+} concentration in the incubation mixture and in the gradient was 2 mM. After centrifugation in a Beckman SW41 rotor at 41,000 rpm at 2° for 3 hr, the tubes were pierced through the bottom and the samples were displaced from the top through a Beckman fraction recovery system. A Gilford spectrophotometer equipped with an 2-mm path flow cell from International Equipment was used to follow the absorbance of the fractions at 260 nm, except in Fig. 2 where each fraction was measured in a Zeiss spectrophotometer.

Pancreatic RNase (Worthington Biochemical Corp.) activity was assayed by measurement of the trichloroacetic acid-soluble nucleotides produced from [14C]RNA (isolated from Erlich ascites tumor cells grown in the presence of [14C]-
formate) after incubation at 37° for 10 min with 0.4 μg/ml of RNase.

**RESULTS**

The total binding of \(^{35}S\)Met-tRNA\(_f\) to reticulocyte ribosomes bearing endogenous mRNA is not significantly affected by pactamycin or NaF, but is inhibited by aurintricarboxylic acid (Table 1). The synthesis of a functional initiation complex, as judged by its ability to form \(^{35}S\)Met-puromycin, however, is impaired by both pactamycin and NaF, provided that these agents are present during complex formation. If it is added after most of the active complex has been formed, pactamycin is not able to interfere significantly with the puromycin reaction (Table 1 and Fig. 1). These results are in agreement with those found with wheat-embryo ribosomes and tobacco mosaic virus RNA as messenger (9).

The formation of an active 80S initiation complex proceeds via a 40S ribosomal subunit-mRNA-Met-tRNA\(_f\) intermediate to which a 60S ribosomal subunit joins (15, 16). The overall process requires initiation factors and GTP. The analysis of binding reactions similar to those described in Table 1 by sucrose density gradient centrifugation shows that formation of the puromycin-reactive 80S initiation complex (Fig. 2A, and D) is prevented by pactamycin (Fig. 2B and E), and that this activity of the drug is associated with a pronounced increase in the amount of \(^{35}S\)Met-tRNA\(_f\) bound to the smaller initiation complex, which sediments slightly further down the gradient (about 50S) than the 40S ribosomal subunit. On the other hand, if the addition of pactamycin is delayed until after most of the active 80S complex has been formed, there is only a slight inhibition of \(^{35}S\)Met-puromycin formation (Fig. 2C and F). It should be noted, however, that the small amount of \(^{35}S\)Met-tRNA\(_f\) bound to the 80S initiation complex in the presence of pactamycin (Fig. 2B) is poorly releasable by puromycin (Fig. 2E). Pactamycin does not in itself cause the 80S complex to dissociate, since the pattern of binding found in Fig. 2C is identical to that in a reaction lacking pactamycin (not shown) that was stopped at the time the antibiotic was added in Fig. 2C.

Although in the control incubation a small peak of \(^{35}S\)Met-tRNA\(_f\) is found in an area on the gradient corresponding to the dimer region (Fig. 2A), in the presence of pactamycin the predominant fast-moving radioactive peak is neither in the monomer or the dimer region, but is between the two regions (about 95 S) (Fig. 2B). While the amount of this particle varies in different experiments for unexplained reasons, its location 3-4 fractions earlier on the gradient than the dimer region is highly reproducible. This material is not produced when pactamycin is added late in the binding reaction (Fig. 2C). Its position on the sucrose gradient suggests that it represents a hybrid ("1.5 mer") between a monosome (ribosome couple on a strand of mRNA) and a pactamycin-inactivated 40S ribosomal subunit bearing \(^{35}S\)Met-tRNA\(_f\) located on the initiation site of the mRNA. Such ribosomal structures have been found under various conditions (15, 17), especially in the presence of NaF (17). Additional support for the notion that pactamycin produces initiation-inactivated 1.5 mers to which the 60S ribosomal subunit fails to join comes from experiments with puromycin or pancreatic RNase. The addition of puromycin to the reaction after binding results in the disappearance of radioactivity (about 2500 cpm) from the putative 1.5 mer region of the gradient, and a concomitant increase (about 2500 cpm) in the amount of radioactivity found in the smaller initiation complex (Fig. 2E). This result is the expected one, since puromycin should lead to the release of the uninvolved ribosomal couple from the mRNA, while the inactivated ribosomal subunit with its \(^{35}S\)Met-tRNA\(_f\) remains attached. By contrast, \(^{35}S\)methionine associated with ribosome monomers and dimers is released to the top

**Table 1. Effect of pactamycin and other agents on Met-tRNA\(_f\) binding to reticulocyte ribosomes and on Met-puromycin formation**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>(^{35}S)Met-tRNA(_f) binding cpm bound (%) inhibition</th>
<th>Met-puromycin formation cpm (%) inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7823 (---)</td>
<td>3433 (---)</td>
</tr>
<tr>
<td>Pactamycin (1 μM)</td>
<td>766 (78)</td>
<td>2652 (14)</td>
</tr>
<tr>
<td>Pactamycin (1 μM) at 7 min</td>
<td>665 (81)</td>
<td>2495 (27)</td>
</tr>
<tr>
<td>Pactamycin (2 μM) at 7 min</td>
<td>267 (92)</td>
<td>852 (75)</td>
</tr>
<tr>
<td>Pactamycin (10 μM)</td>
<td>2347 (70)</td>
<td></td>
</tr>
<tr>
<td>NaF (10 mM)</td>
<td>5602 (28)</td>
<td></td>
</tr>
<tr>
<td>ATA (0.2 mM)</td>
<td>7190 (8)</td>
<td></td>
</tr>
</tbody>
</table>

Binding incubations (7 min, 23°) in a total volume of 0.1 ml contained 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.5 mM GTP, 100 mM KCl, 2 mM MgCl\(_2\), 2.24 A\(_{260}\) units of 0.5 M KCl-washed ribosomes, 0.17 A\(_{260}\) units of \(^{35}S\)Met-tRNA (46,000 cpm), and 278 μg of ribosomal wash protein. For Met-puromycin synthesis, 0.5 mM puromycin was added to the binding incubation at 7 min, and incubation was continued for 10 min at 30°. Pactamycin, NaF, and aurintricarboxylic acid (ATA) were present from the start of the reaction unless otherwise indicated.
of the gradient as \[^{[4S]}\text{Met-puromycin}\] (Fig. 2D and F). Furthermore, RNase treatment of binding reactions results in disappearance of the putative radioactive 1.5 mer, with little change or a small increase in the radioactivity associated with the 80S complex (Fig. 3). Because there is a large amount of radioactivity in the region of the smaller initiation complex in incubations containing pactamycin, compared with the small peak of the putative 1.5 mer, it has not been possible to consistently account for the counts lost in the putative 1.5 mer region as appearing in the smaller complex region. Therefore, several attempts have been made to isolate the putative 1.5 mer by centrifugation, and to treat it with RNase before further centrifugation on a sucrose gradient. Although we have been able to demonstrate the unequivocal formation of radioactive smaller initiation complexes by this procedure, the recoveries have been repeatedly low, on the order of 20–30%. On the other hand, treatment of labeled 80S complexes with RNase does not decrease their radioactivity, and no labeled smaller initiation complexes are produced.

We have obtained evidence that pactamycin alters the structure of the smaller initiation complex. Such structures are considerably more resistant to degradation by pancreatic RNase than are similarly sedimenting particles that form at early times in the absence of pactamycin (Fig. 3B and D). This protective action of pactamycin is not due to inhibition of RNase itself, as shown in experiments with \[^{[14C]}\text{RNA}\] derived from ascites tumor cells.

The sensitivity of Met-puromycin formation to pactamycin depends on the concentration of Mg\(^{2+}\) (Fig. 4). When the binding reaction takes place at higher Mg\(^{2+}\) concentrations, the pactamycin effect is considerably decreased. Sucrose gradients (Fig. 5) confirm that formation of the functional 80S initiation complex at 5 mM Mg\(^{2+}\) is inhibited to the same small extent as is the synthesis of Met-puromycin. If the Mg\(^{2+}\) concentration is increased to 5 mM after binding in the presence of pactamycin is over, there is a partial reversal of inhibition of the puromycin reaction and formation of the 80S initiation complex.

![Figure 2](image1.png)

**Fig. 2.** Sucrose gradient centrifugation of \[^{[4S]}\text{Met-tRNA}\] bound to washed reticulocyte ribosomes, and the effect of pactamycin. Reaction mixtures (0.1 ml) containing 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM GTP, 100 mM KCl, 2 mM MgCl\(_2\), 2.9 A\(_{260}\) units of ribosomes, 400 \(\mu\)g of ribosomal wash protein, and 0.39 A\(_{260}\) units of \[^{[4S]}\text{Met-tRNA}\] (61,400 cpm) were incubated for 14 min at 30°C in the presence or absence of pactamycin (1 \(\mu\)M), and layered onto sucrose gradients. After centrifugation, the gradients were fractionated; the fractions were diluted to 1 ml with the gradient buffer before absorbance at 260 nm was measured in a Zeiss spectrophotometer. Centrifugation was from left to right. (A) Control without pactamycin. (B) Pactamycin present from the beginning. (C) Pactamycin added at 7 min. (D, E, and F) 0.5 mM puromycin was added at 7 min to incubations identical to A, B, C, respectively.

![Figure 3](image2.png)

**Fig. 3.** Effect of RNase treatment on \[^{[4S]}\text{Met-tRNA}\] bound at 4 min to reticulocyte ribosomes. Incubation mixture, in a total volume of 0.1 ml, contained 2.5 A\(_{260}\), units of ribosomes, 600 \(\mu\)g of ribosomal wash protein, and 0.29 A\(_{260}\) units of \[^{[4S]}\text{Met-tRNA}\] (63,000 cpm). Other conditions are similar to those in Fig. 2. The tubes were incubated for 4 min at 30°C and chilled to 0°C; RNase (1 \(\mu\)g/ml) was added as indicated. After standing at 0°C for 15 min, the mixture (90 \(\mu\)l) was layered on the sucrose gradient. (A) Control. (B) Control after treatment with RNase. (C) Binding in the presence of pactamycin (2 \(\mu\)M) from the start of the incubation. (D) C after RNase treatment.
DISCUSSION

These experiments provide additional support for the concept that pactamycin produces a nonfunctional initiation complex with reticulocyte ribosomes bearing endogenous mRNA (2, 5). A similar conclusion has been reached from experiments with wheat embryo ribosomes and tobacco mosaic virus RNA as messenger (9). Our results on sucrose density gradients, however, differ from those reported by Seal and Marcus (9) and Weeks and Baxter (17) with wheat embryo ribosomes and viral mRNA. These workers report that pactamycin does not lead to the accumulation of the smaller ribosomal initiation complex. In the only sucrose gradient data actually presented from the wheat embryo system, the Mg\textsuperscript{2+} concentration in the binding reaction was relatively high, 3.6 mM (17). It is not clear, however, whether the difference in results is due to differences in reaction conditions, or to basic differences between reticulocyte and wheat embryo ribosomes, mRNA, or initiation factors. The data from Fig. 2B and E are compatible with the formation of an inactive 80S complex in the presence of pactamycin in the reticulocyte system but, in contrast to the results in the wheat embryo system, the altered larger initiation complex readily dissociates into its subunit components.

It is clear that pactamycin, which binds only to the smaller ribosomal subunit (1), alters the structure of the smaller initiation complex in such a way that it is resistant to the action of pancreatic RNase. Whether antibiotic bound to the 40S ribosomal subunit induces a structural change in the complex that affords greater protection to the attached mRNA or tRNA is not known. Furthermore, it is of interest that the smaller initiation complex, which is a normal intermediate in peptide-chain initiation, is sensitive to mild RNase treatment, whereas the larger initiation complex, like the pactamycin-modified smaller initiation complex, is resistant (Fig. 3). This finding raises the possibility that the protective phenomena due to the attachment of the 60S ribosomal subunit or to the binding of the antibiotic have a common structural basis, an induced change in the conformation of the smaller initiation complex.

The lack of puromycin-reactivity of ribosome-bound Met-tRNA\textsubscript{f} found at 2 mM Mg\textsuperscript{2+} in the presence of pactamycin in the system used in this paper can be accounted for by the accumulation of smaller initiation complexes (either free or as oligoribosomes). What, if any, relation these phenomena have to the inhibition of peptide chain initiation found in more complex systems such as reticulocytes and their lysates remains to be elucidated. The specificity of pactamycin for initiation may rest rather on the fact that the antibiotic binds to free 40S ribosomal subunits or run-off ribosomes, but not to ribosomes (in polysomes) already engaged in chain elongation (1). In this way the step in the protein synthesis sequence blocked by pactamycin need not be one peculiar to initiation but may be one involved in elongation, such as translocation or acceptor site binding. Thus, only the last ribosome to join a polysome structure would be unable to move down the mRNA. Such an effect would be viewed grossly (as measured by polysome degradation and globin chain completion) as one on "initiation," even though it is actually "elongation" that is being affected. We are studying the effect of pactamycin on the formation of the initial di- and tripeptides of globin in an effort to settle these points.

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Fig. 4. Effect of magnesium concentration on pactamycin inhibition of Met-puromycin synthesis. The reaction conditions are similar to those described in the legend for Fig. 1, except that the concentration of the initiation factors was 400 \mu g and puromycin (0.5 mM) and pactamycin (1 \mu M) were present from the start of the reaction. O, Control; *, with pactamycin; \Delta, % inhibition by pactamycin.

Fig. 5. Effect of pactamycin on puromycin-sensitive initiation complex formation at 5 mM magnesium. Incubation conditions and centrifugation analysis were the same as in Fig. 2, except that 0.29 \text{ A}_{260} units of [\text{35S}]Met- tRNA (63,000 cpmp) was used. (A) Control. (B) Pactamycin (1 \mu M). (C) Puromycin (0.5 mM) added to control at 7 min. (D) Puromycin (0.5 mM) added to B at 7 min.