BIOCHEMICAL STUDY OF MINOSAMINOMYCIN IN RELATION TO
THE KASUGAMYCIN GROUP ANTIBIOTICS

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Minosaminomycin is structurally related to kasugamycin and inhibits protein synthesis in mycobacteria. It also inhibits phage f2 RNA-directed protein synthesis in a cell-free system of Escherichia coli by 50% at 2 x 10^{-7} M. It is 100-times more potent than kasugamycin in this system. At 10^{-7} M minosaminomycin inhibits EF-T dependent binding of aminoacyl-tRNA to ribosomes by 50%. This effect is markedly diminished if minosaminomycin is added to the assay system after a brief incubation of ribosomes with mRNA. Like kasugamycin, minosaminomycin preferentially inhibits the initiation of protein synthesis directed by phage f2 RNA in vitro and does not cause miscoding. Ribosomes from kasugamycin-resistant mutants Ksg A and Ksg C were as sensitive to minosaminomycin as those from each parent strain. In spite of the strong inhibitory activity of minosaminomycin manifested in cell-free systems of E. coli, this compound inhibits the growth of the organism itself only slightly. This discrepancy could be ascribed to impermeability, as E. coli cells with modified permeability show greater sensitivity to minosaminomycin. There is no indication that the antibiotic is inactivated in E. coli cells. On the basis of these results, the structural features of these antibiotics essential for interaction with ribosomes and for permeability into the cells are discussed.

Minosaminomycin (MSM) was isolated as an inhibitor of the growth of mycobacteria by Hamada et al. and its structure was determined by Inuma et al. Based on its structural similarity to kasugamycin (KSM), we presumed that MSM was also an inhibitor of initiation of protein synthesis. It was found that MSM inhibited the synthesis of protein in mycobacteria. Since the cell-free protein synthesizing system of E. coli is well established and was found to be strongly inhibited by MSM, this system was utilized for a detailed study of the mode of action of MSM. The reason for the lack of activity of MSM on E. coli was investigated. Structure-activity studies defining features essential for binding to ribosomes and those essential for cell permeation are considered in this paper.

Materials and Methods

In vitro protein synthesis and related reactions.

In vitro protein synthesis using S-30 extracts of E. coli Q13 and phage f2 RNA as mRNA was conducted as previously reported. In brief, a reaction mixture contained in 100 µl, 50 mM Tris-HCl, pH 7.8, 3 mM ATP, 0.2 mM GTP, 60 mM NH4Cl, 2 mM phosphoenol pyruvate, 5 µg of pyruvate kinase, 2 mM dithiothreitol, 18 µM[14C]Val (233 mCi/m mole), 20 µM each of 19 amino acids, 10 mM Mg(OAc)2, 0.6 A260 of uncharged tRNA, 0.2 mg protein equivalent of S-30, a desired amount of a test compound and 1.7 A260 of phage f2 RNA. After incubation at 37°C for 20 minutes, radioactivity in the protein fraction was determined.

Initiation-free polysomes were prepared as follows: Cells of E. coli B in early exponential phase...
(0.2 A660, 80 ml culture) were harvested on crushed ice and submitted to gentle lysis as described by Flessel.6) The lysate (1 ml) containing polysomes was spun at 130,000 g for 17 hours in a centrifuge tube containing 1 ml of 70% sucrose in TMN (10 mM Tris-HCl (pH 7.8)-10 mM Mg(OAc)2-50 mM NH4Cl) and 3 ml of 30% sucrose in TMN. The polysomes were suspended by gentle mixing in TMN containing 6 mM mercaptoethanol, divided into small portions, and stored at -90°C until use.

KSM-resistant strains; Ksg A (E. coli TPR201) and its parent strain (E. coli PR7)7) were kindly supplied by Dr. J. E. Davies, University of Wisconsin, and Ksg C (E. coli AB312 Ksg 2) and its parent strain (E. coli AB312)8 by Dr. M. Yoshikawa, University of Tokyo.

Plasmolysis of E. coli was performed according to the method of Gros et al.9 with a minor modification; washed cells of E. coli Q13 were suspended in 2M sucrose-0.01 M Tris-HCl, pH 8.0, at a density of about 2 × 10^13 cells/ml and were kept at 30°C for 10 minutes (plasmolyzed cells). For comparison, a portion of washed cells was submitted to the same procedures except that sucrose was omitted from the buffer (untreated cells). After chilling, the cells were freed from the sucrose by centrifugation.

Results

Effect of MSM on Synthesis of Cellular Macromolecules in Mycobacteria

The effect of MSM on the synthesis of proteins, cell walls and nucleic acids by exponentially growing cells of mycobacteria is shown in Fig. 1. MSM strongly inhibited the incorporation of amino acids into acid-insoluble cell material, in other words, inhibited protein synthesis. Other macromolecular synthesis was relatively unaffected.

Permeability Barrier to MSM in E. coli

The antibiotic spectrum of MSM is relatively narrow. From the following observations, the resistance of E. coli to MSM seems to be due to a permeability barrier. Protein synthesis in E. coli cells subjected to plasmolysis, thus modifying their permeability, was inhibited more strongly than that in untreated cells (Table 1). However, complete inhibition could not be obtained. The plasmolysis was confirmed by the inhibitory effect of actinomycin D on the RNA synthesis in these cells. Attempts to alter cellular permeability to MSM by other means, such as osmotic shock10 or treatment with phenethyl alcohol11) was unsuccessful. Incubation of a MSM solution with a sonicate of cells of E. coli did not lower significantly its antibiotic titer (data not shown). Therefore, it is

![Fig. 1. Effect of minosaminomycin on macromolecular synthesis in Mycobacterium 607](image)
unlikely that the resistance of E. coli could be due to the enzymatic inactivation of MSM.

Effect of MSM on Poly(U)-Directed Protein Synthesis with an E. coli Cell-free System

The above experiments suggested that a cell-free protein synthesizing system of E. coli would be sensitive to MSM, as the permeability barrier is removed. This was tested by determining the effect on poly(U)-directed protein synthesis in vitro and the result is shown in Table 2. MSM, but
not KSM or kasugamycin KG-8 (a chemical derivative of KSM, see Table 5 for the structure), was active. MSM inhibited this reaction as strongly as kanamycin, a member of a bactericidal group of aminosugar antibiotics. In view of the marked difference of the activity between MSM and the other two members of this family (KSM and kasugamycin KG-8), it seemed possible that MSM might have some other mode of action, for instance, miscoding. This was tested by measuring the poly(U)-directed incorporation of \(^{14}\text{C}\)isoleucine into

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<th>Table 3. Effect of minosaminomycin on protein synthesis with initiation-free polysomes</th>
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<tr>
<td><strong>Concentration (m)</strong></td>
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<tr>
<td>Minosaminomycin</td>
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<tr>
<td>Kasugamycin</td>
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<td>Chloramphenicol</td>
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Protein synthesis with initiation-free polysomes (see Methods) was performed as reported by Tai et al.\(^{15}\). Under these conditions, protein synthesis leveled off after 20 minutes of incubation at 37°C. The reaction speed, determined in the initial 5 minutes, paralleled the concentration of polysomes up to 1.0 \(A_{260}\) mm/0.1 ml of the assay mixture. Magnesium concentration (6~20 mm) did not influence the inhibitory effect of minosaminomycin. The assay was conducted with 100 \(\mu\)l reaction mixtures (10 mm \(\text{Mg(OAc)}_2\) and 0.3 \(A_{260}\) polysomes) at 37°C for 20 minutes. \(^{14}\text{C}\)Val incorporation was determined.

![Fig. 2. Effect of minosaminomycin on the amount of ribosome-bound peptides and released peptides (in vitro system directed by phage f2 RNA)](image)

For analysis of the amounts of ribosome-bound peptides and released peptides, the volume of the reaction mixture (phage f2 RNA-directed protein synthesis; see Methods) was increased to 200 \(\mu\)l. After incubation, a 100 \(\mu\)l portion of each mixture was submitted to sucrose density gradient centrifugation analysis which was conducted as reported previously\(^{4}\). (○) no antibiotic. (●) with 2 \times 10\(^{-4}\)m minosaminomycin. The arrows indicate the time of minosaminomycin addition.
Fig. 4. Drug-sensitivities of ribosomes from kasugamycin-sensitive and kasugamycin-resistant E. coli
(a) Crude ribosomes, sedimented at 30,000~100,000 g, were obtained from E. coli strains TPR201 (Ksg A) and its parent PR7. In vitro protein synthesis directed by phage f2 RNA as mRNA was performed as described in methods with the following modifications; S30 was replaced by crude ribosomes (1.8 A260 and 1.3 A260 for TPR201 and PR7, respectively) and 0.1 mg protein of S105 from E. coli Q13, and 17.5 μM each of 19 amino acids and 1.2 A260 of phage f2 RNA were used.

(b) S30 fractions were prepared from E. coli strain AB312 Ksg 2 (Ksg C) and its parent AB312, and in vitro protein synthesis was performed with phage f2 RNA as mRNA (see Methods).

<table>
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<tr>
<th>Messenger</th>
<th>% Inhibition</th>
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<td>Poly (G, U)</td>
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Table 4. Effect of various antibiotics on binding of f[14C]Met-tRNA to ribosomes

Binding of f[14C]Met-tRNA to ribosomes in the presence of an appropriate template was determined according to the method of DUNNOFF and MAITRA. A reaction mixture contained in 100 μl, 50 mM Tris-HCl, pH 7.5, 80 mM NH4Cl, 6 mM Mg(OAc)2, 4 mM dithiothreitol, 0.1 mg of crude initiation factors, a template (0.1 A260 of AUG, 0.2 A260 of poly(G, U) or 1.7 A260 of phage f2 RNA), 1.3 A260 of washed ribosomes, 0.15 μM f[14C]Met-tRNA (255 mCi/m mole), and antibiotic and water to volume. Incubation conditions were varied depending on template; 25°C for 10 minutes for AUG, 37°C for 10 minutes for poly(G, U) and 37°C for 15 minutes for phage f2 RNA. Radioactivity retained on nitrocellulose filters was determined.
polypeptide chains (Table 2). MSM showed no miscoding effect. As reference, the miscoding activity of negamycin \(^{13}\), determined under the same conditions, is shown. Similar results were obtained in poly(C)- and poly(A)-directed incorporation experiments.

Inhibition by MSM of Initiation of Protein Synthesis

*In Vitro*

Protein synthesis can be divided into 3 distinct steps; initiation, elongation and termination. To determine on which step MSM acts, an *in vitro* protein synthesizing system directed by phage f2 RNA was utilized. Protein synthesis was initiated in the absence of MSM for 3 minutes. MSM was then added and incubation was continued for 10 or 20 minutes. Incorporation of \(^{14}C\) valine into ribosome-bound and released peptides were determined at the times indicated. As shown in Fig. 2, after addition of MSM, the amount of ribosome-bound peptides decreased rapidly, while that of released peptides continued to increase at a rate comparable to that of the control. These results indicated that MSM primarily inhibited initiation, but had no or a slight effect on the elongation and termination processes. This observation was confirmed by a separate experiment in which the effect of MSM on protein synthesis by initiation-free polysomes was determined. As shown in Table 3, MSM had a minor effect under conditions where ribosomes in the polysomal structure only run off from mRNA but do not initiate another round of protein synthesis. In addition, MSM did not inhibit the peptidyltransferase reaction; MSM at \(10^{-4}\)M did not inhibit release of peptidyl puromycin in a reaction mixture containing \(^{3}H\)puromycin and derived monosomes carrying nascent peptides \(^{13}\) under conditions where \(6 \times 10^{-4}\)M chloramphenicol showed 77% inhibition (data not shown).

**Effect of MSM on Binding of \[^{14}C\]Met-tRNA to Ribosomes**

In the initiation process of protein synthesis, \[^{14}C\]Met-tRNA binding to ribosomes is directed by an RNA template containing the AUG or GUG sequence. The effect of MSM on the binding of \[^{14}C\]Met-tRNA to ribosomes in the presence of AUG, poly(G,U) or phage f2 RNA was determined. MSM at \(10^{-4}\)M showed 70~90% of inhibition depending on templates, as shown in Table 4. On
Effect on Binding of \([^{14}\text{C}]\text{Phe-tRNA}\) to Ribosomes

The data shown so far indicates that MSM primarily inhibits the initiation but has only a slight effect on the elongation and termination processes in protein synthesis (Fig. 2, Tables 3, 4). Therefore, one would presume that MSM has very little or no effect on binding of aminoacyl-tRNA to ribosomes. However this was not the case. As shown in Fig. 3, MSM strongly inhibited this reaction; 50\% inhibition was observed at 10^{-7} M MSM. However, the inhibitory effect of MSM was markedly reduced if ribosomes and mRNA were incubated briefly before the assay was initiated (Fig. 3). It should be noted that the effect of tetracycline was unchanged under these conditions. These results indicate that ribosomes bound to mRNA are much less sensitive to MSM than free ribosomes. This mechanism must explain the specific inhibition by MSM of the initiation process, in which free ribosomes play a role, and the lack of inhibition of the elongation processes where ribosomes are in association with mRNA.

Sensitivity to MSM of Ribosomes from KSM Resistant Strains of E. coli

Considering the similarity in the structure and mode of action of MSM and KSM, we presumed that the ribosomes from a KSM resistant strain of E. coli would be insensitive to MSM as well as to KSM. This possibility was tested in an in vitro protein synthesizing system including ribosome preparations from either KSM resistant or sensitive strains. As Fig. 4 shows, ribosomes from the KSM-resistant strains required about 10 times the concentration of this antibiotic for equivalent inhibition than ribosomes from the sensitive strains. However, the KSM sensitive and resistant ribosome preparations showed the same sensitivity to MSM. An analogous result was obtained for kasugamycin KG-8. The minute structural differences of these compounds could be recognized by ribosomes, although these compounds appear to share a common site of action on the ribosome, leading to inhibition of the initiation step.

Relationship between Structure and In Vitro Activity

Various members and derivatives of the KSM family, including MSM, were tested for their effect on ribosomes with a cell-free protein synthesizing system directed by phage f2 RNA. The results are expressed in Table 5 as % inhibition of \([^{14}\text{C}]\text{Leu}\) incorporation exerted by each compound at the indicated concentrations. Since MSM was a much stronger inhibitor than KSM in vitro, the equatorial N-blocked amino group on C-1, as in MSM, seems to be a preferred structure to the axial hydroxyl group on the same carbon, as in KSM. Since minobiosamine, in which the equatorial amino group is free, was only slightly active, the blocked amino group is the preferred structure. With regard to the substituent on C-4', a free amino group, as in MSM, is sufficient for in vitro activity. However, a basic side chain in that position, as in kasugamycin KG-8, presumably increases the permeability of Gram-negative bacteria because kasugamycin KG-8 is about 10-times as active in inhibiting the growth of E. coli as KSM. The free amino group on C-2' is not an essential structure for inhibition of ribosome function, since kasugamycin TK-27 showed some activity. All compounds lacking the inositol moiety were inactive. Based on these observations, we predict that a potent antimicrobial agent may be obtained by introduction of appropriate side chains on minobiosamine. The function of the presumptive side chains on the 4' and 2' amino groups may be involved in permeation while...
those on the amino group at the C-1 position may be involved both in permeation and in association with ribosomes.

Discussion

The antibiotic MSM, structurally related to KSM, inhibited protein synthesis in *E. coli* cell-free systems more strongly than KSM. MSM inhibited preferentially the initiation step of protein synthesis with no or slight inhibition of elongation and termination (Fig. 2, Tables 3, 4). It neither inhibited the puromycin reaction and the translocation reaction\(^{13}\) (data not shown) nor caused miscoding (Table 2). In these respects MSM resembled KSM. However, their modes of action were not identical. For instance, MSM inhibited the poly(U)-directed binding of Phe-tRNA to ribosomes (“Phe-tRNA binding”) as strongly as it inhibited the binding of fMet-tRNA to ribosomes in the presence of poly (G,U), phage f2 RNA or AUG (“fMet-tRNA binding”) (Fig. 3, Table 4). In contrast, KSM was a ten-fold more potent inhibitor of “fMet-tRNA binding” than “Phe-tRNA binding” (Fig. 3, Table 4). In view of the lack of inhibition by MSM of the elongation process, its inhibition of “Phe-tRNA binding” seemed contradictory. This question was answered by the finding that, if ribosomes and poly(U) were preincubated, MSM did not inhibit “Phe-tRNA binding” so strongly; MSM seems to act on free ribosomes but not on ribosomes which are in association with mRNA. Consistent with this notion was the observation that inhibition of polyphenylalanine synthesis by MSM (Table 2) was abolished if the assay was started with a preformed AcPhe-tRNA-ribosome-poly(U) complex (data not shown). The exact nature of the MSM-resistant poly(U)-ribosome complex, formed in 10mM magnesium is not known. In contrast to the results with the poly(U) systems, MSM inhibited “fMet-tRNA binding” even when the ribosomes were preincubated with poly(G,U) or not (data not shown). This supports the fact that MSM has a specific action on the initiation process.

Ribosomes from *E. coli* Ksg A and Ksg C mutants were still sensitive to MSM (Fig. 4). With kasugamycin KG-8, a resistant mutant of *E. coli* was isolated (*E. coli* Q13 KG-8 R). Ribosomes from this mutant compared with those from the parent strain were 10 times more resistant to KG-8 but not to KSM or MSM (data not shown). The lack of co-resistance between these structurally related compounds may reflect detailed differences in their modes of action on ribosomes.

The antimicrobial activity of a drug inhibiting protein synthesis is not solely dependent on its affinity for the ribosome but also on its permeability into and metabolism in target cells. The lack of antimicrobial activity of MSM to *E. coli* appears to be due to impermeability (Table 1). Since MSM inhibited the growth of mycobacteria by inhibiting protein synthesis (Fig. 1), it indicated that MSM was permeable to this organism. It is presumed that hydrophilic drugs such as aminosugar antibiotics are taken up into cells through some specific transport systems of normal metabolites. Identification of the transport mechanism is important for future studies on chemical modification of this group of antibiotics.

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