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Cross-Linking of Streptomycin to the 30S Subunit of *Escherichia coli* with Phenyldiglyoxal†

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ABSTRACT: [^{3}H]Dihydrostreptomycin was covalently linked to the 30S subunit of *Escherichia coli* K12A19 with the bifunctional cross-linking reagent phenyldiglyoxal. The cross-linking was abolished under conditions that prevent the binding of streptomycin, which indicates that the cross-linking occurs at the specific binding site of streptomycin. The cross-linking involved 16S RNA and the ribosomal proteins S1, S5, S11, and S13. This suggests that the streptomycin binding site is located in the upper part of the 30S subunit, facing the 50S subunit. Unexpectedly, the same extent and pattern of cross-linking were observed with the 30S subunits from a streptomycin-resistant mutant. We have shown previously that streptomycin induces conformational changes in the ribosomes from sensitive bacteria but not from streptomycin-resistant mutants. From this and from the results in the present study, it is suggested that the binding of streptomycin to streptomycin-sensitive ribosomes is a two-step reaction wherein an initial loose interaction at the antibiotic binding site is followed by a conformational rearrangement of the ribosomal particle. The second step would tighten the association with streptomycin and cause interference with protein synthesis. That step would be lacking in streptomycin-resistant mutants.

The aminoglycoside antibiotic streptomycin interacts with the ribosome and interferes with several steps of protein synthesis in bacteria [reviewed in Vazquez (1979) and Wallace et al. (1979)]. In spite of extensive studies, the molecular mechanisms involved in the interaction of streptomycin with the ribosome are still poorly understood. The identification of the ribosomal components involved in the binding of streptomycin should provide new information on these mechanisms.

Streptomycin binds to the 30S subunit of *Escherichia coli* at a single binding site (Chang & Flaks, 1972; Schreiner & Nierhaus, 1973; Grisé-Miron & Brakier-Gingras, 1982). Affinity analogues of streptomycin have been used to probe the streptomycin binding site (Cooperman, 1980; Girshovich et al., 1976; Pongs & Erdmann, 1973; Pongs et al., 1974). In this study, we used a bifunctional cross-linking agent as a novel way to characterize the binding site of streptomycin. In this procedure, the specific complex between streptomycin and the 30S subunit can be formed before initiation of the cross-linking reaction. This is an advantage over the affinity labeling procedures which may generate unspecific reactions depending upon the reactivity of the modified streptomycin. We chose the cross-linking reagent phenyldiglyoxal, which specifically reacts with guanosine in single-stranded RNA and the guanidine groups of arginine residues in proteins and has been successfully used to make RNA–RNA and protein–RNA cross-links in the bacterial ribosome (Wagner & Garrett, 1978;

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Chiam & Wagner, 1983). This reagent has the potential to cross-link streptomycin through its guanidine residues to either ribosomal RNA or proteins. The results reported here show that streptomycin is cross-linked by phenyldiglyoxal primarily to 16S RNA but also to ribosomal proteins. We have identified the cross-linked proteins and propose that the streptomycin binding site is located in the upper part of the 30S subunit, at the interface between the ribosomal subunits.

Materials and Methods

Reagents. Phenyl-1,4-diglyoxal was obtained from Molecular Probes. Ribonuclease A (EC 3.1.27.5), ribonuclease T1 (EC 3.1.27.3), and ribonuclease-free proteinase K (EC 3.4.21.14) were purchased from Boehringer. Streptomycin sulfate, Coomassie blue G-250 and R-250, urea "ultra-pure" and [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris)[1] were from Sigma. Bluensomycin sulfate was a gift from the Upjohn Co. Acrylamide and bis(acrylamide) were from Bio-Rad. Sodium dodecyl sulfate was from the British Drug House. Iodogen was purchased from Pierce Chemical Co. Sodium [^{3}H]borohydride (specific activity 8.1 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq), Aquasol, Protosol, and Econofluor were from New England Nuclear. [^{125}I]Iodine (carrier free, 100 mCi/mL) was from Amersham. [^{3}H]Dihydrostreptomycin was prepared by reduction of streptomycin with sodium [^{3}H]borohydride as described by Chang & Flaks (1972). Its specific activity was 4 Ci/mmol.

Preparation of 30S Ribosomal Subunits. 70S ribosomes from *E. coli* K12A19 (ribonuclease I$^-$) and from a spontaneous streptomycin-resistant derivative (mutant Sm^r-53) were isolated as tight couples from slow-cooled bacteria by standard procedures (Grisé-Miron & Brakier-Gingras, 1982). Ribosomes were dissociated into subunits by lowering the magnesium concentration to 1 mM, and ribosomal subunits were fractionated by zonal centrifugation (28 000 rpm, 20 h) through a 10–40% (w/v) linear sucrose gradient in a Beckman Al-14 zonal rotor. Contamination of the 30S subunits by 50S subunits was less than 3%, as assessed by sucrose gradient centrifugation.

Cross-Linking Reactions. The 30S ribosomal subunits were incubated for 15 min at 37 °C in the cross-linking reaction buffer (100 mM sodium cacodylate, 200 mM potassium chloride, 10 mM magnesium acetate, and 6 mM β-mercaptoethanol, pH 8) with [^{3}H]dihydrostreptomycin added at a molar ratio of one per ribosomal particle. The concentration of potassium chloride was chosen to prevent nonspecific random interactions between streptomycin and the 30S subunit, while allowing the formation of the stable complex at the single high-affinity streptomycin binding site (Schreiner & Nierhaus, 1973). Phenyldiglyoxal was dissolved in dioxane at a concentration of 10 mg/mL immediately before use, diluted with the reaction buffer, and added to the incubation medium, at a molar ratio of 5000 per ribosomal particle. In a typical cross-linking reaction, the final concentration of the 30S ribosomal subunits was 5 A_{260} units/mL [1 A_{260} unit equals 74 pmol of 30S (Kearney & Moore, 1983)]. The 30S ribosomal subunits were incubated with phenyldiglyoxal for 45 min at 37 °C. The reaction was stopped by adding arginine (at a final concentration of 20 mM), and the [^{3}H]dihydrostreptomycin which was not covalently bound to the 30S subunits was displaced by addition of an excess (200-fold) of unlabeled dihydrostreptomycin.

The 30S subunits treated with phenyldiglyoxal cosedimented with control untreated 30S subunits in a sucrose gradient (results not shown), indicating that the treatment with phenyldiglyoxal does not induce gross structural changes in the ribosomal particles.

Study of the Cross-Linking of Dihydrostreptomycin to 30S Subunits by Millipore Filtration. Millipore filtration was used as a rapid assay to assess the cross-linking reaction. After cross-linking [^{3}H]dihydrostreptomycin to the 30S subunits with phenyldiglyoxal, the [^{3}H]dihydrostreptomycin which was not covalently linked was chased as described above, and the 30S ribosomal subunits were filtered on Millipore nitrocellulose filters (type HA; pore size, 0.22 μm) (Grisé-Miron & Brakier-Gingras, 1982). Controls without the cross-linking agent or without the chasing step were run in parallel. The radioactivity on the filters was counted in a toluene-based scintillation fluid. The efficiency of counting was 18%.

Distribution of Covalently Bound Dihydrostreptomycin between RNA and Proteins in 30S Subunits. After [^{3}H]dihydrostreptomycin was cross-linked to the 30S subunits, the reaction mixture was divided into three portions, and the 30S subunits were precipitated by addition of 2 volumes of ethanol. One sample was dissolved in the RNA digestion buffer (0.1 M Tris-HCl, 0.01 M EDTA, and 3 M urea, pH 6) and digested for 2 h at 37 °C with a mixture of pancreatic RNase and RNase T1 (each added at a ratio of 1 μg per 20 μg of RNA). The second sample was dissolved in the protein digestion buffer (0.1 M Tris-HCl, 0.01 M EDTA, 0.05 M NaCl, and 0.2% $NaDodSO_4$, pH 6) and digested for 2 h at 37 °C with proteinase K (added at a ratio of 1 μg per 20 μg of protein). The third sample was dissolved indifferently in the protein or the RNA digestion buffer and incubated in the absence of digestion enzymes. After the digestion reaction, RNA or the proteins were precipitated twice with ethanol. The undigested control was precipitated under the same conditions. The precipitates were dissolved and counted in Aquasol. The efficiency of counting was 35%.

Identification of 30S Proteins Cross-Linked to Streptomycin. After the cross-linking reaction, the 30S subunits were digested with a mixture of pancreatic RNase and RNase T1, and the proteins were fractionated by using two different one-dimensional electrophoresis systems: an 18% polyacrylamide/urea slab gel at an acidic pH, with an acrylamide:bis(acrylamide) ratio of 1 to 0.014 (Knopf et al., 1975), and a 12.5% polyacrylamide/$NaDodSO_4$/urea slab gel at neutral pH, with an acrylamide:bis(acrylamide) ratio of 1 to 0.033 (Swank & Munkres, 1971).[2] The gels were then stained either with 0.08% (w/v) Coomassie blue G-250 in 12.5% (w/v) trichloroacetic acid for acidic urea gels or with 0.2% (w/v) Coomassie blue R-250 in methanol/acetic acid/water (4:1:5 v/v) for $NaDodSO_4$ gels. All the electrophoresis experiments were done in duplicate. After the completion of electrophoresis, the slab gels were cut into slices. One sample was used to determine which slices contained proteins labeled with [^{3}H]dihydrostreptomycin: the slices were incubated at 37 °C overnight in a 5% Protosol solution in Econofluor and counted. The duplicate sample was used to identify the proteins in the

[1] Abbreviations: DHSm, dihydrostreptomycin; EDTA, ethylenediaminetetraacetic acid; Iodogen, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril; $NaDodSO_4$, sodium dodecyl sulfate; RNase, ribonuclease; Sm^s, streptomycin sensitive; Sm^r, streptomycin resistant; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

[2] The system of Swank and Munkres was preferred to the more commonly used polyacrylamide/$NaDodSO_4$ system of Laemmli (1970) because the high pH of the latter system promotes the cleavage of the bond between arginine residues and phenyldiglyoxal, and the release of [^{3}H]dihydrostreptomycin.

Table I: Cross-Linking of [^{3}H]Dihydrostreptomycin with Phenyldiglyoxal to the 30S Subunits from *E. coli*[a]

conditions of reaction			
bacterial strain	phenyldiglyoxal	addition of unlabeled DHSm	binding of [^{3}H]DHSm (cpm)
Sm^s	absent	no	61278
Sm^s	absent	yes	196
Sm^s	present	no	63716
Sm^s	present	yes	3902
Sm^r	absent	no	808
Sm^r	absent	yes	204
Sm^r	present	no	3738
Sm^r	present	yes	3624

[a] The binding of [^{3}H]dihydrostreptomycin to the 30S subunits was assessed by Millipore filtration as described under Materials and Methods. Unlabeled dihydrostreptomycin, added in excess, chases the [^{3}H]dihydrostreptomycin which is not covalently linked to the 30S subunits with phenyldiglyoxal. Results correspond to 3 A_{260} units of 30S subunits. They are the means of three independent experiments. The standard deviation on the means was ±15%. The efficiency of counting was 18%. The specific activity of [^{3}H]dihydrostreptomycin was 8800 dpm/pmol.

radioactive slices. This identification was performed as follows: the proteins were extracted from the gels and incubated for 2 h at pH 9 at 37 °C. This treatment releases [^{3}H]dihydrostreptomycin and regenerates the unmodified proteins (Takahashi, 1968). The proteins were then iodinated with 0.1 mM KI containing 100 μCi of [^{125}I]iodine and Iodogen (Tolan et al., 1980). They were identified by coelectrophoresis with a mixture of unlabeled 30S proteins in the two-dimensional polyacrylamide/urea acidic gel electrophoresis system of Knopf et al. (1975). Gels were dried and exposed at room temperature for 3–4 days to Fuji RX X-ray films.

Results

Cross-Linking of Streptomycin to the 30S Subunit. [^{3}H]Dihydrostreptomycin was bound to the 30S subunit from the Sm^s strain *E. coli* K12A19 in the absence or the presence of phenyldiglyoxal, and non-cross-linked streptomycin was chased by adding an excess of unlabeled antibiotic. The amount of [^{3}H]dihydrostreptomycin bound to the 30S subunit was assessed by Millipore filtration assays. The results are presented in Table I. They confirm that [^{3}H]dihydrostreptomycin bound tightly to the 30S particles and that this binding was reversible, since [^{3}H]dihydrostreptomycin was efficiently chased by an excess of unlabeled dihydrostreptomycin (Chang & Flaks, 1972). However, after treatment with phenyldiglyoxal, a significant fraction of [^{3}H]dihydrostreptomycin could not be chased and remained irreversibly bound to the 30S subunits, indicating that phenyldiglyoxal can cross-link [^{3}H]dihydrostreptomycin to the 30S subunits. When [^{3}H]dihydrostreptomycin was added to the 30S subunits from the Sm^r derivative of *E. coli* K12A19, mutant Sm^r-53, the amount of bound antibiotic was very low in the absence of phenyldiglyoxal, confirming that the antibiotic does not bind tightly to the Sm^r 30S subunits. However, in the presence of phenyldiglyoxal, the amount of irreversibly bound [^{3}H]dihydrostreptomycin markedly increased and reached the same value in the Sm^r 30S subunits as in the Sm^s 30S subunits. This shows that [^{3}H]dihydrostreptomycin can be cross-linked to the same extent to the Sm^r and to the Sm^s subunits. Other Sm^r mutants from our collection were also investigated (data not shown) and confirmed this unexpected finding.

Since [^{3}H]dihydrostreptomycin can be covalently attached to both the Sm^r and the Sm^s 30S subunits, the cross-linking reaction might be nonspecific. We could not determine whether cross-linked [^{3}H]dihydrostreptomycin could mimic the effect of streptomycin on protein synthesis, since the treatment with phenyldiglyoxal inactivates the 30S subunits (Chiam & Wagner, 1983). Therefore, to ascertain that the cross-linking reaction did not occur at random sites, the cross-linking reaction was carried out under conditions which prevent the interaction of streptomycin with its specific binding site. This was accomplished either by raising the KCl concentration above 500 mM or by adding bluensomycin, a drug which competes for streptomycin binding site (Chang & Flaks, 1972). The cross-linking reaction was abolished under both of these conditions (data not shown). This indicates that the covalent binding of [^{3}H]dihydrostreptomycin to the 30S subunits does occur at the specific binding site of the antibiotic.

The distribution of the cross-linked [^{3}H]dihydrostreptomycin between 16S RNA and the protein moiety of the 30S subunits was assessed by digesting either the proteins with proteinase K or the RNA with a mixture of RNases. The remaining ribosomal component was then precipitated with ethanol, and the radioactivity bound to the precipitate was counted. The results indicated that about 75% (±7%) of the cross-linking involved the 16S RNA with both the Sm^s and Sm^r strains.

Identification by Polyacrylamide Gel Electrophoresis of Proteins Cross-Linked to Streptomycin. After [^{3}H]dihydrostreptomycin was cross-linked to the 30S subunits, the ribosomal proteins were extracted and fractionated by polyacrylamide gel electrophoresis in order to determine which proteins were linked to streptomycin. Figure 1 shows the fractionation of the 30S ribosomal proteins by the different gel electrophoresis systems which are used in this study. Figure 2 presents an example of the distribution of radioactivity among the proteins from the Sm^s *E. coli* strain, when fractionated by one-dimensional polyacrylamide/urea gel electrophoresis according to Knopf et al. (1975). There were four major peaks of radioactivity, designated I, II, III, and IV. Except for slight variations in the relative height of the peaks, this pattern was reproducible from one experiment to the next. Since treatment with phenyldiglyoxal may alter the electrophoretic mobility of ribosomal proteins, the identity of the proteins present in the radioactive peaks could not be unambiguously established by simple determination of their mobility in the gels, except for peak I. Peak I contains protein S1, which is unambiguously identified since no other ribosomal protein migrates in that region of the gel. The proteins of the three other peaks were extracted from the gels and incubated at pH 9, which regenerates the unmodified proteins. They were then radioiodinated and identified by two-dimensional gel electrophoresis. The proteins found in the peaks were, respectively, S4, S5, and S7 in peak II, S9, S10, and S11 in peak III, and S12 and S13 in peak IV (Figure 3).

Since more than one protein was present in three of the radioactive peaks observed with one-dimensional polyacrylamide/urea gel electrophoresis, it was not clear whether all of these proteins are labeled with [^{3}H]dihydrostreptomycin. When proteins labeled with [^{3}H]dihydrostreptomycin were fractionated by another procedure, one-dimensional polyacrylamide/NaDodSO$_4$ gel electrophoresis according to Swank & Munkres (1971), four peaks of radioactivity were observed (Figure 4). Using the method of identification described above, we found that peak I contained S1; peak II, S5; peak III, S8 and S11; and peak IV, S13 and S14 (Figure 5). In the case of peak III, the presence of S11 and not S9, which comigrate when submitted to two-dimensional gel electrophoresis, was further established by fractionating the radioiodinated proteins by one-dimensional polyacrylamide/Na-

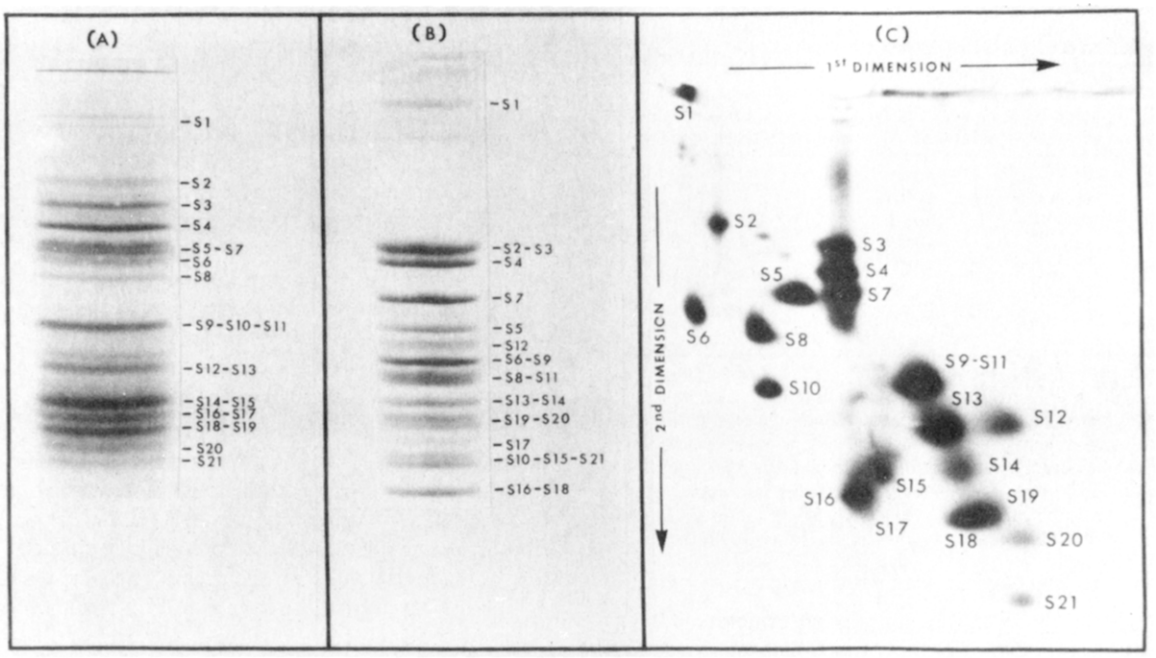

FIGURE 1: Typical examples of the fractionation of the 30S ribosomal proteins from *E. coli* K12A19 by (A) one-dimensional polyacrylamide/urea acidic gel electrophoresis according to Knopf et al. (1975), (B) one-dimensional polyacrylamide/$NaDodSO_4$ gel electrophoresis according to Swank & Munkres (1971), or (C) two-dimensional polyacrylamide/urea acidic gel electrophoresis according to Knopf et al. (1975). Proteins were stained with Coomassie blue. The identity of the proteins in the stained bands of the gel systems (A) and (B) was established by determining their position of migration in the two-dimensional gel system of Knopf et al. (C). This determination was performed as follows: proteins were extracted from the gels, radioiodinated, and submitted to two-dimensional gel electrophoresis in the presence of unlabeled carrier proteins. Two-dimensional gel electrophoresis does not resolve S9 from S11. In this case, the position of migration of proteins S9 and S11 in the one-dimensional gels was established by using purified S9 and S11.

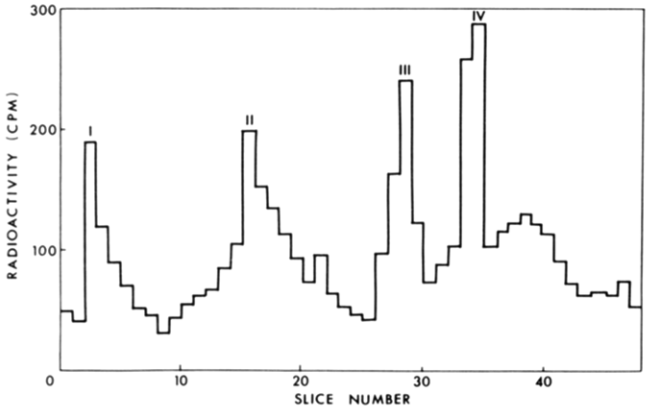

FIGURE 2: One-dimensional polyacrylamide/urea acidic gel electrophoresis analysis of 30S proteins from *E. coli* labeled with [^{3}H]-dihydrostreptomycin. Reported counts per minute are for protein extracted from 10 A_{260} units of 30S subunits.

$DodSO_4$ gel electrophoresis which resolves S11 from S9 (Figure 1B).

Table II summarizes which proteins were found in the radioactive peaks in the two different one-dimensional electrophoresis systems. Those proteins associated with radioactive peaks in both systems are assumed to be those which are cross-linked to [^{3}H]dihydrostreptomycin. Therefore, we can conclude from the results in Table II that proteins S1, S5, S11, and S13 are cross-linked to streptomycin.

We have also attempted to analyze the proteins labeled with [^{3}H]dihydrostreptomycin by two-dimensional gel electrophoresis. However, when that method was used, the yield of radioactivity was very low. Nevertheless, it was possible to detect some radioactivity around proteins S5, S9/S11, and S13. This supports the conclusions drawn from one-dimensional gel electrophoresis. As to S1, which remains near the origin in two-dimensional gel electrophoresis, no radioactivity could be unequivocally ascribed to this protein when this fractionation procedure was used.

Table II: Identification of the 30S Proteins from *E. coli* Cross-Linked to [^{3}H]Dihydrostreptomycin with Phenyldiglyoxal after Fractionation by Polyacrylamide Gel Electrophoresis

system A[a]	system B[a]
peak I: S1	peak I: S1
peak II: S4, S5, S7	peak II: S5
peak III: S9, S10, S11	peak III: S8, S11
peak IV: S12, S13	peak IV: S13, S14

[a] Proteins cross-linked to [^{3}H]dihydrostreptomycin were fractionated either by one-dimensional 18% acrylamide/urea gel electrophoresis (system A) or by 12.5% acrylamide/$NaDodSO_4$ gel electrophoresis (system B), as illustrated in Figures 2 and 4, respectively. The proteins present in the radioactive fractions were identified as described in the text and in the legends to Figures 3 and 5.

Ribosomal proteins were also fractionated after cross-linking [^{3}H]dihydrostreptomycin to the 30S subunits from the Sm^r mutant. The patterns of labeling were identical with those obtained with the proteins from the Sm^s strain (data not shown).

Discussion

Our results show that streptomycin can be cross-linked with phenyldiglyoxal primarily to 16S RNA and also to the 30S proteins S1, S5, S11, and S13. The cross-linking is specific since it does not occur in the presence of bluensomycin or at a high salt concentration, conditions which prevent the formation of the complex between streptomycin and the 30S subunit.

This is the first report that protein S11 is located in the streptomycin binding site while other approaches have already pointed to the involvement of proteins S1, S5, and S13 in the binding of streptomycin. Proteins S1 and S13 have been labeled with an affinity analogue of streptomycin [M. A.

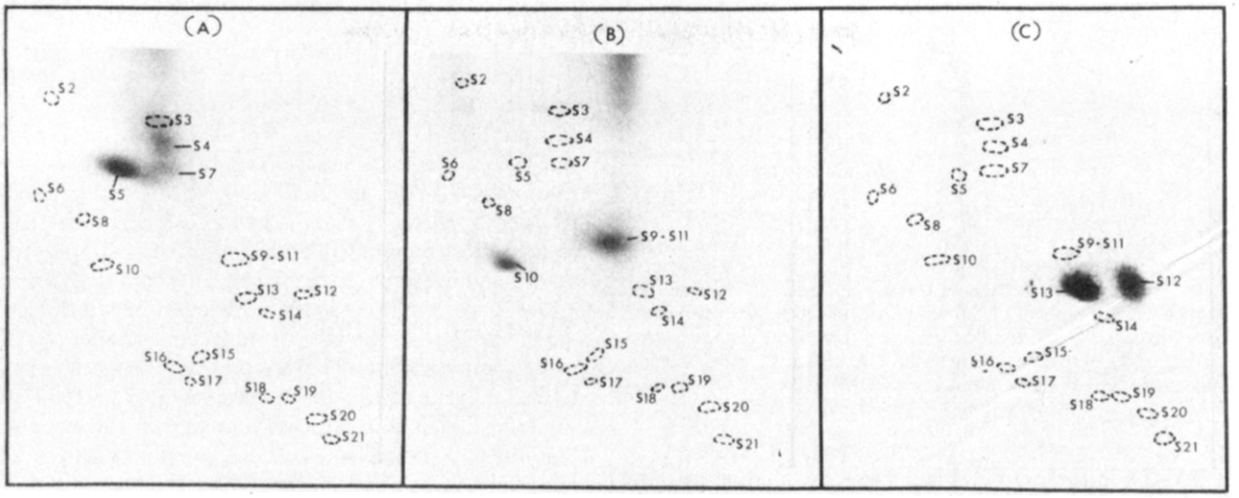

FIGURE 3: Identification by two-dimensional polyacrylamide/urea gel electrophoresis of the proteins present in the radioactive fractions indicated in Figure 2. Panels A, B, and C are autoradiograms which correspond to fractions II, III, and IV of Figure 2, respectively. The autoradiograms were obtained as described under Materials and Methods. The proteins in the radioactive fractions were radioiodinated and submitted to two-dimensional gel electrophoresis in the presence of carrier proteins. The proteins were stained with Coomassie blue G-250 (see the detailed pattern in Figure 1C), and the autoradiograms were superimposed on the stained gel. Stained proteins are indicated by broken circles.

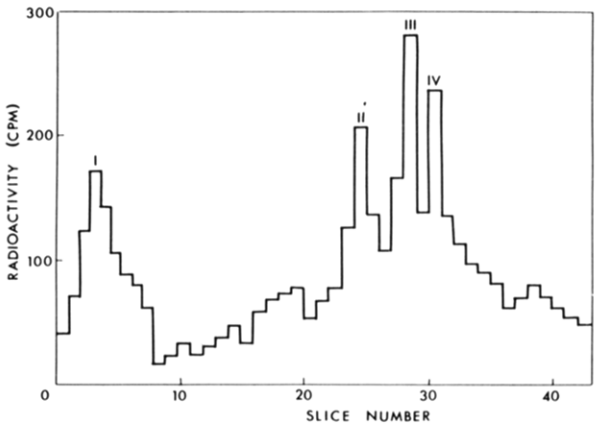

FIGURE 4: One-dimensional polyacrylamide/NaDodSO$_4$ gel electrophoresis analysis of 30S proteins from *E. coli* labeled with [^{3}H]-dihydrostreptomycin. Reported counts per minute are for protein extracted from 10 A_{260} units of 30S subunits.

Luddy and B. S. Cooperman, unpublished results; quoted in Cooperman (1980)]. However, in a previous study, we have shown that the omission of S1 does not impair the interaction of streptomycin with the ribosome (Grisê-Miron & Brakier-Gingras, 1982). Binding studies with protein-deficient cores of the 30S subunits have shown that the addition of protein S5 enhances the binding of streptomycin (Schreiner & Nierhaus, 1973) and mutations in S5 alter the ribosomal response to streptomycin, causing reversion from streptomycin dependence [Hasenbank et al., 1973; reviewed in Stöffler & Wittmann (1977)]. In addition, binding studies using equilibrium dialysis have shown that these mutations in S5 increase the affinity of the ribosome for streptomycin (Böck et al., 1979).

Three of the four proteins cross-linked to streptomycin, S5, S11, and S13, are known to be near-neighbors since cross-links can be formed between S5 and S13, and between S11 and S13 (Traut et al., 1980; Lambert et al., 1983). In addition, proteins S11 and S13 have been identified as subunit interface proteins by cross-linking studies between ribosomal subunits (Cover et al., 1981; Lambert & Traut, 1981). Protein S5 is probably also an interface protein, since it is found in small amounts in purified 50S subunits (Kenny et al., 1979). This suggests that the streptomycin binding site is located at the interface between the ribosomal subunits. Proteins S11 and S13 were also chemically or photochemically cross-linked to the initiation factors IF-2 and IF-3 (MacKeen et al., 1980; Boileau et al., 1983), which suggests a possible overlap between the streptomycin binding site and the binding site for the initiation

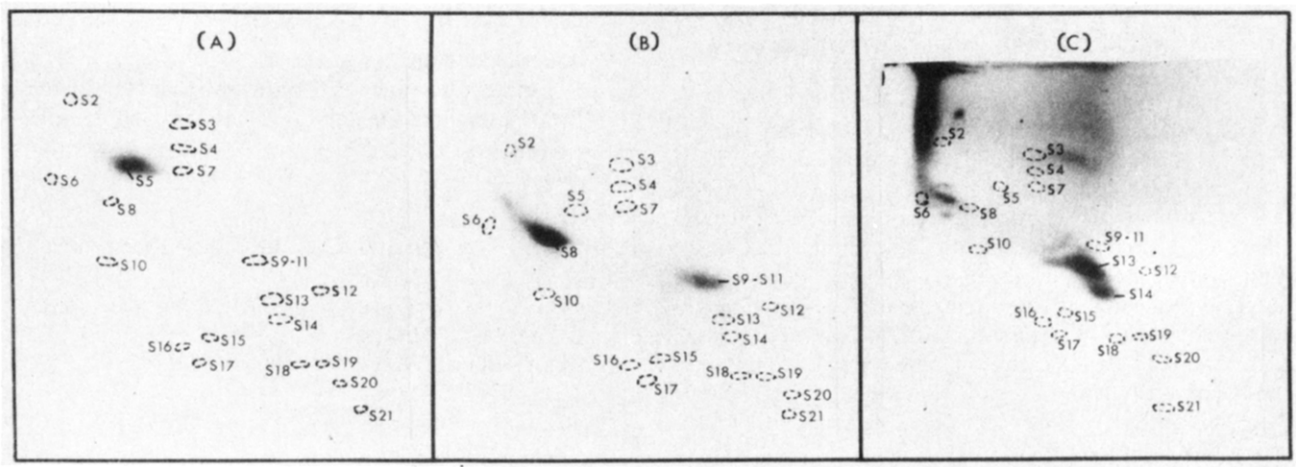

FIGURE 5: Identification by two-dimensional polyacrylamide/urea gel electrophoresis of the proteins present in the radioactive fractions indicated in Figure 4. See details in the legend to Figure 3 and under Materials and Methods. Panels A, B, and C are autoradiograms which correspond to fractions II, III, and IV of Figure 4, respectively.

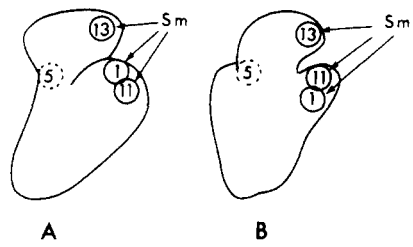

FIGURE 6: 30S subunit models of Lake and Kahan (A) and Stöffler (B), showing the internal surface of the subunit and the position of the antigenic determinants of the proteins cross-linked to streptomycin. The dashed lines indicate a localization away from the viewer, on the external face of the subunit, while the solid lines indicate a localization on the internal face of the subunit.

factors. This is supported by a previous report that streptomycin and the initiation factors compete for binding to the 30S subunit (Lelong et al., 1972). No cross-link has ever been found between protein S1 and proteins S5, S11, or S13 (Boileau et al., 1981), but several lines of evidence indicate that part of S1 is close to the binding site of the initiation factors [reviewed in Grunberg-Manago (1980) and Boileau et al. (1983)] and, therefore, should be in the neighborhood of proteins S11 and S13.

Other studies with different affinity analogues of streptomycin have identified S4, S7, S14, and S16/S17 as being located in the binding site for streptomycin (Pongs & Erdmann, 1973; Pongs et al., 1974; Girshovich et al., 1976). These results do not, however, contradict our findings, since cross-linking studies have demonstrated that S4 and S7 are proximal to S5 and S13 (Expert-Benzançon et al., 1977; Traut et al., 1980; Lambert et al., 1983). Protein S14 was not cross-linked to any of the proteins which we have identified in the streptomycin binding site. However, it interacts with S5 since reconstitution studies have shown that it enhances the stimulation of streptomycin binding by S5 (Schreiner & Nierhaus, 1973). It also interacts with protein S1 since it stimulates the binding of S1 to protein-deficient 30S subunits (Laughrea & Moore, 1978). Another independent approach, based on the masking of antigenic determinants with the corresponding antibody fragment (Lelong et al., 1974), has suggested that the binding site for streptomycin should be located near S19. This result is also consistent with our data. Indeed, cross-linking studies have demonstrated that S13 and possibly S11 are proximal to S19 (Expert-Benzançon et al., 1977; Traut et al., 1980; Lambert et al., 1983).

Figure 6 relates the cross-linking data reported in this study to the models of the 30S subunit as determined by Lake and Kahan and by Stöffler, using immune electron microscopy (Lake, 1980; Kahan et al., 1981; Winkelmann et al., 1982; Stöffler et al., 1980; Stöffler & Stöffler-Meilicke, 1983). The antigenic determinant for protein S13 is in the head of the 30S subunit, at the subunit interface, and antigenic determinants for S1 and S11 have been mapped on the so-called platform of the 30S subunit. The antigenic determinant for protein S5 is however located at the rear, on the external face of the subunit, but as mentioned above, it is known that part of S5 must also be located at the subunit interface, in the neighborhood of protein S13 (Kenny et al., 1979; Traut et al., 1980). Our cross-linking data locate the binding site of streptomycin in an important functional region, which contains the binding site for messenger RNA, for the initiation factors, and for parts of the aminoacyl- and peptidyl-tRNA binding sites [reviewed in Cooperman (1980) and Ofengand (1980)]. This location is consistent with the interference of streptomycin with several steps of protein biosynthesis.

Our cross-linking studies have also indicated that streptomycin can be cross-linked as well to streptomycin-sensitive and to streptomycin-resistant particles and that the cross-linking involves the same set of proteins. Studies with Millipore filtration (Chang & Flaks, 1972; Table I of this study) have shown that streptomycin binds tightly to streptomycin-sensitive ribosomes but does not bind tightly to streptomycin-resistant ribosomes. Protein S12 is known to control the ribosomal response to streptomycin and is mutated in streptomycin-resistant and in streptomycin-dependent mutants [Ozaki et al., 1969; Birge & Kurland, 1969; reviewed in Stöffler & Wittmann (1977)]. In the present study, S12 was not cross-linked to streptomycin with phenyldiglyoxal although it is proximal to S13 (Traut et al., 1980; Lambert et al., 1983), which suggests that it is not directly located in the streptomycin binding site. Our results rule out a previous suggestion that mutations in protein S12 might hinder the accessibility of streptomycin to its binding site (Schreiner & Nierhaus, 1973), since streptomycin can be cross-linked to streptomycin-resistant ribosomes. Using spin-labeled and fluorescent probes, we have observed (Brakier-Gingras et al., 1974, 1976; Noreau et al., 1980; P. Melançon and L. Brakier-Gingras, unpublished results) that streptomycin induces conformational changes in streptomycin-sensitive but not in streptomycin-resistant ribosomes. These previous studies and the results in the present report are consistent with the following interpretation: the binding of streptomycin to streptomycin-sensitive 30S subunits would occur in a two-step reaction. An initial interaction at the streptomycin binding site would be followed by a conformational rearrangement of the ribosomal particle, which simultaneously would tighten the association between the antibiotic and its target and cause interference with protein synthesis. In this model, mutations to streptomycin resistance would not abolish the initial interaction between the ribosome and the antibiotic but would interfere with the second step of the binding, the conformational rearrangement. According to this interpretation, protein S12 should occupy a key position in the neighborhood of the streptomycin binding site, which controls the ability of the ribosome to undergo conformational changes upon the binding of the drug.

Finally, our results stress a major involvement of 16S RNA in the binding of streptomycin. This finding is in agreement with previous reports from Gorini and his collaborators (Biswas & Gorini, 1972; Garvin et al., 1974), who proposed, on the basis of dialysis binding studies, that 16S RNA provides the binding site for streptomycin. To further characterize the binding site of streptomycin, it will be important to identify which parts of 16S RNA can be cross-linked to the antibiotic.

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