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Cross-Linking of Streptomycin to the 50S Subunit of *Escherichia coli* with Phenyldiglyoxal†

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Received November 30, 1984

ABSTRACT: [^{3}H]Dihydrostreptomycin was covalently linked to the 50S subunit of *Escherichia coli* K12A19 with the bifunctional cross-linking reagent phenyldiglyoxal. The cross-linking was abolished under conditions that prevent the specific interaction of streptomycin with the ribosome. The binding primarily involved the ribosomal RNA and also a limited number of proteins, namely, L2, L6, and L17. This suggests that the binding domain for streptomycin is close to the peptidyl transferase center, in the valley between the central protuberance and the wider lateral protuberance of the 50S subunit. This domain faces the binding domain for streptomycin which we have previously characterized on the 30S subunit [Melançon, P., Boileau, G., & Brakier-Gingras, L. (1984) *Biochemistry 23*, 6697–6703]. Our results indicate that the 50S subunit is involved in the binding of streptomycin to the bacterial ribosome, in addition to the 30S subunit which is generally considered as the specific target of the antibiotic. They are consistent with the occurrence of a single binding site for streptomycin on the ribosome, comprised of regions of both subunits.

Streptomycin binds to a single site on the 70S ribosome of *Escherichia coli* and perturbs several steps of protein synthesis [reviewed in Vazquez (1979) and Wallace et al. (1979)]. It is generally assumed that the 30S subunit is the target of streptomycin in the 70S ribosome, since streptomycin binds tightly to the 30S subunit but not to the 50S subunit (Chang & Flaks, 1972; Schreiner & Nierhaus, 1973; Grisé-Miron & Brakier-Gingras, 1982) and resistance to or dependence on streptomycin results from mutations affecting the 30S protein S12 [Ozaki et al., 1969; Birge & Kurland, 1969; reviewed in Stöffler & Wittmann (1977)]. However, when it binds to the 70S ribosome, streptomycin induces conformational changes in the 50S subunit (Delihas et al., 1975; Martinez et al., 1978; Tritton, 1978), suggesting that it may also directly interact with this subunit.

In a previous study (Melançon et al., 1984), we characterized the binding site of streptomycin on the 30S subunit using the bifunctional cross-linking reagent phenyldiglyoxal. This binding site was located on the head of the 30S subunit, at the interface with the 50S subunit. In the present study,

† Supported by a grant from the Medical Research Council of Canada.

0006-2960/85/0424-6089$01.50/0

we have used phenyldiglyoxal to investigate the interaction between streptomycin and the 50S subunit. Our results show that streptomycin can be cross-linked by phenyldiglyoxal to the 50S subunit. The cross-linking primarily involved the ribosomal RNA but also involved a limited set of proteins. These proteins define a single binding domain for the antibiotic, which faces its binding domain on the 30S subunit.

Materials and Methods

Reagents. Phenyl-1,4-diglyoxal was obtained from Molecular Probes. Pancreatic 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 and methyl *p*-hydroxybenzimidate hydrochloride were 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 50S Ribosomal Subunits. The 50S subunits originated from *E. coli* K12A19 (ribonuclease I$^-$). The 70S ribosomes were isolated as tight couples from slowly cooled cells as described previously (Grisé-Miron & Brakier-Gingras, 1982), and the 50S subunits were isolated by zonal centrifugation as described (Melançon et al., 1984).

Cross-Linking of Streptomycin to 50S Subunits. The cross-linking procedures employed in this study follow those described previously in detail in the study of the cross-linking of streptomycin to the 30S subunits with phenyldiglyoxal (Melançon et al., 1984). The 50S 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. 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 50S ribosomal subunits was 10 A_{260} units/mL [1 A_{260} unit equals 39 pmol of 50S subunit (Kearney & Moore, 1983)]. The 50S 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 50S subunits was displaced by addition of an excess (200-fold) of unlabeled dihydrostreptomycin.

In some assays, [^{3}H]dihydrostreptomycin was cross-linked to the 70S ribosomes, under the same experimental conditions as those described for the 50S subunits. After the cross-linking reaction, the 70S ribosomes were dissociated into subunits by lowering the magnesium concentration and the subunits fractionated by centrifugation through a sucrose gradient.

The 50S subunits treated with phenyldiglyoxal cosedimented with control untreated 50S subunits in a sucrose gradient (results not shown), indicating that the treatment with phenyldiglyoxal did not induce gross structural changes in the ribosomal particles.

Study of Cross-Linking of Dihydrostreptomycin to 50S Subunits by Millipore Filtration. Millipore filtration was used as a rapid assay to assess the cross-linking reaction. After [^{3}H]dihydrostreptomycin was cross-linked to the 50S subunits with phenyldiglyoxal, the [^{3}H]dihydrostreptomycin which was not covalently linked was chased as described above, and the 50S 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 and 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 50S Subunits. After [^{3}H]dihydrostreptomycin was cross-linked to the 50S subunits, the reaction mixture was divided into three portions, and the 50S 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 in either the protein or the RNA digestion buffer and incubated in the absence of RNases or proteinase K. After the digestion, the proteins or RNA was 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 50S Proteins Cross-Linked to Streptomycin. After the cross-linking reaction, the 50S 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 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]dihydro-

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Iodogen, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril; $NaDodSO_4$, sodium dodecyl sulfate; RNase, ribonuclease; 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 [^{3}H]dihydrostreptomycin and the ribosomal proteins.

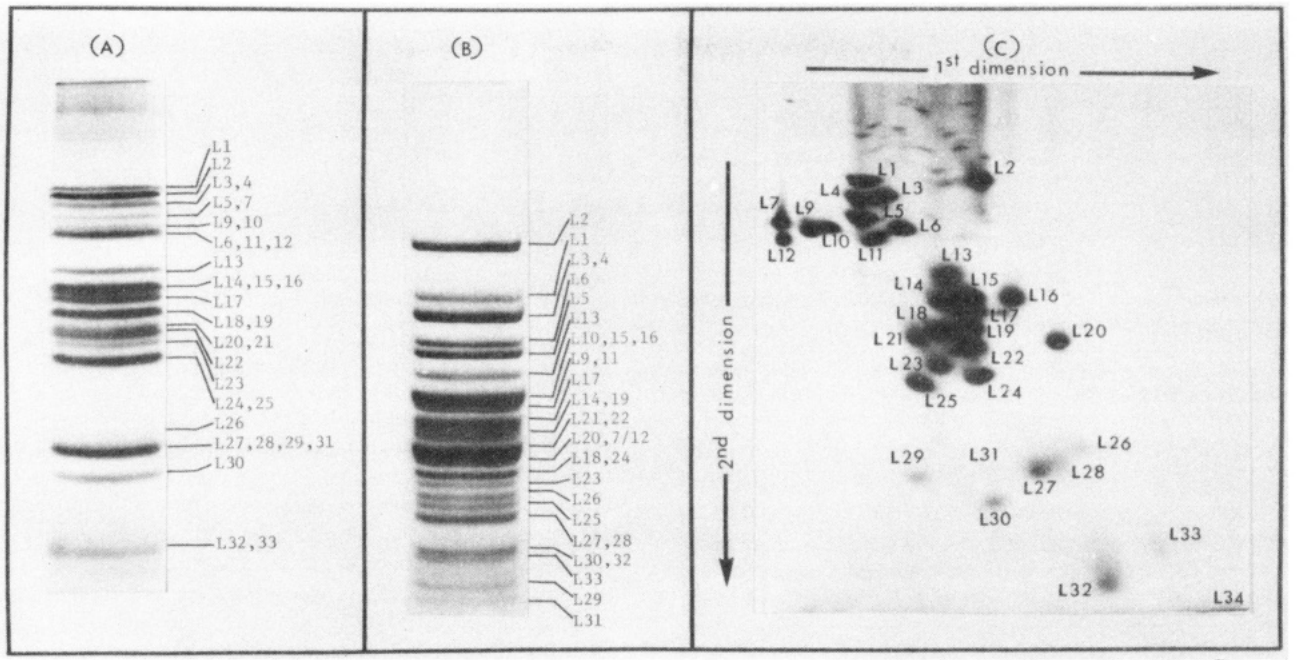

FIGURE 1: Typical examples of the fractionation of 50S ribosomal proteins from *E. coli* K12A19 by (A) one-dimensional polyacrylamide/urea acidic gel electrophoresis according to Knopf et al. (1975), (B) one-dimensional polyacrylamide/$NaDodSO_4$ gel electrophoresis according to Swank & Munkres (1971), and (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.

streptomycin 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 50S 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 X-ray films.

RESULTS

Cross-Linking of Streptomycin to 50S Subunits. Equimolar amounts of [^{3}H]dihydrostreptomycin and 50S subunits from *E. coli* were incubated with or without phenyldiglyoxal, and non-cross-linked streptomycin was then chased by adding an excess of unlabeled antibiotic. The amount of [^{3}H]dihydrostreptomycin bound to the 50S subunits was assessed by Millipore filtration assays. The results are presented in Table I. They confirm that [^{3}H]dihydrostreptomycin did not bind tightly to the 50S subunits in the absence of phenyldiglyoxal (Chang & Flaks, 1972). However, after treatment with phenyldiglyoxal, a significant fraction of [^{3}H]dihydrostreptomycin (about 1% of the input) remained bound to the 50S subunits and could not be chased by adding an excess of dihydrostreptomycin, indicating that phenyldiglyoxal can cross-link [^{3}H]dihydrostreptomycin to the 50S subunits. A very similar fraction of input [^{3}H]dihydrostreptomycin was covalently linked to the 30S subunits under similar conditions (Melançon et al., 1984).

To determine if the binding of [^{3}H]dihydrostreptomycin to the 50S subunits was specific, cross-linking was performed under conditions which prevent the specific interaction of the antibiotic with the ribosome. This was done by raising the concentration of KCl to 700 mM in the cross-linking buffer or by adding bluensomycin, a closely related antibiotic which competes for the streptomycin binding site on the ribosome (Chang & Flanks, 1972). Bluensomycin, when present, was added in a 1 to 1 or a 2 to 1 molar ratio with respect to [^{3}H]dihydrostreptomycin. The cross-linking of [^{3}H]dihydrostreptomycin was abolished at the high ionic strength or decreased in proportion to the added amount of bluensomycin (data not shown). This supports the interpretation that the covalent binding of [^{3}H]dihydrostreptomycin to the 50S subunits is specific.

The partition of the cross-linked [^{3}H]dihydrostreptomycin between the ribosomal RNA and the protein moiety of the 50S subunits was assessed by digesting the proteins with proteinase K or the RNA with a mixture of RNases. The remaining ribosomal component was then precipitated with ethanol, and the precipitate was counted. The results indicated that about 75% (±7%) of streptomycin was cross-linked to ribosomal RNA (data not shown).

Identification by Polyacrylamide Gel Electrophoresis of Proteins Cross-Linked to Streptomycin. After [^{3}H]dihydrostreptomycin was cross-linked to the 50S subunits, the ribosomal proteins were extracted and fractionated by polyacrylamide gel electrophoresis in order to determine which proteins were linked to streptomycin. Figure 1 illustrates the fractionation of the 50S ribosomal proteins from *E. coli* by the different gel electrophoresis systems which are used in this study. Figure 2 presents an example of the distribution of the radioactivity among the 50S ribosomal proteins, when fractionated by one-dimensional polyacrylamide/urea acidic gel electrophoresis according to Knopf et al. (1975). There were three major peaks of radioactivity, designated I, II, and III. Except for slight variations in the relative height of the peaks, this pattern was reproducible. Kinetic assays showed that the radioactivity in these three peaks increased with time to level off after about 30–40 min of reaction (data not shown). There was no lag in the uptake of radioactivity in any of these three peaks. This rules out the possibility that labeling with [^{3}H]dihydrostreptomycin might result from prior alterations of the ribosomal particles by the cross-linking reagent. Since treatment with phenyldiglyoxal may alter the electrophoretic mobility of ribosomal proteins, the identity of the proteins

Table I: Cross-Linking of [^{3}H]Dihydrostreptomycin with Phenyldiglyoxal to 50S Subunits from *E. coli*[a]

conditions of reaction		binding of [^{3}H]dihydrostreptomycin (cpm)
phenyldiglyoxal	addition of unlabeled dihydrostreptomycin	
absent	no	611
absent	yes	167
present	no	3548
present	yes	3382

[a] The binding of [^{3}H]dihydrostreptomycin to the 50S 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 50S subunits with phenyldiglyoxal. Results correspond to 6 A_{260} units of 50S 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.

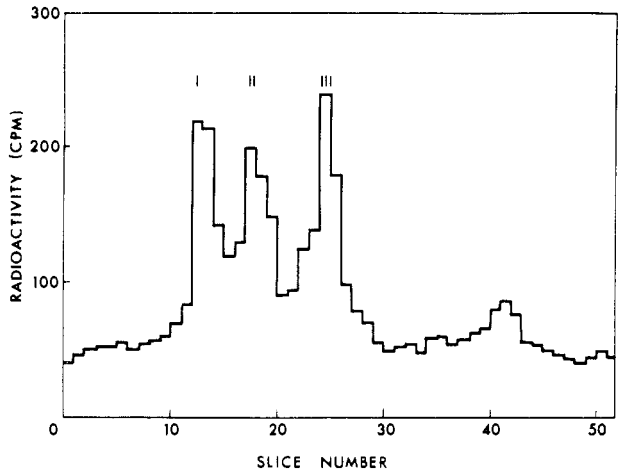

FIGURE 2: One-dimensional polyacrylamide/urea acidic gel electrophoresis analysis of 50S proteins from *E. coli* labeled with [^{3}H]-dihydrostreptomycin. Reported counts per minute are for protein extracted from 20 A_{260} units of 50S subunits.

present in the radioactive peaks could not be unambiguously established from their mobility in the gels. The proteins of the three peaks were extracted from the gels and incubated at pH 9, which regenerates the unmodified proteins (Takahashi, 1968). They were then radioiodinated according to the procedure of Tolan et al. (1980), as described under Materials and Methods, and identified by two-dimensional gel electrophoresis. The proteins found in the peaks were respectively L1 and L2 in peak I, L6, L9, L10, and L11 in peak II, and L14, L15, L16, and L17 in peak III (Figure 3).

Since more than one protein was present in all the radioactive peaks observed with one-dimensional polyacrylamide/urea gel electrophoresis, it was not clear whether all of these proteins were labeled with [^{3}H]dihydrostreptomycin or whether some unlabeled proteins comigrated with the labeled proteins. Furthermore, all the 50S proteins can be readily iodinated, except L7 and L12.[3] L7 and L12 migrate

[3] Since L7 and L12 lack tyrosine and histidine, they cannot be readily iodinated using the standard procedure of Tolan et al. (1980), as indicated under Materials and Methods. A modification of the standard procedure of Tolan et al. (1980), involving a derivatization of the proteins with methyl *p*-hydroxybenzimidate, allows the iodination of proteins which do not contain tyrosine and histidine. However, since this modified procedure is less efficient than the standard procedure, we have used it only to radioiodinate purified L7 and L12 and to determine their position of migration in the one-dimensional gel electrophoresis systems, in the presence of unlabeled carrier proteins (Figure 1A,B).

Table II: Identification of 50S 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: L1, L2	peak I: L2
peak II: L6, L9, L10, L11	peak II: L6
peak III: L14, L15, L16, L17	peak III: L17, L19

[a] Proteins cross-linked to [^{3}H]dihydrostreptomycin were fractionated either by one-dimensional 18% acrylamide/urea acidic 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.

in the area corresponding to peak II in the one-dimensional polyacrylamide/urea gel electrophoresis, and, therefore, they might also be labeled with [^{3}H]dihydrostreptomycin but not be detected. This prompted us to use an alternative procedure of fractionation of the 50S proteins, the one-dimensional polyacrylamide/NaDodSO$_4$ gel electrophoresis according to Swank & Munkres (1971). Under these conditions, three major peaks of radioactivity were observed (Figure 4). We identified the proteins present in each peak as described above. Peak I contained L2, peak II, L6, and peak III, L17 and L19 (Figure 5). There was no radioactivity in the area of migration of L7 and L12, which is below peak III (see Figure 1B). This demonstrates that proteins L7 and L12 were not cross-linked to streptomycin.

Table II summarizes the proteins found in the radioactive peaks, in the two different one-dimensional electrophoresis systems. Those proteins associated with radioactive peaks, in both procedures of fractionation, are assumed to be cross-linked to [^{3}H]dihydrostreptomycin. Therefore, we can conclude from the results in Table II that proteins L2, L6, and L17 are cross-linked to streptomycin.

We have found previously that, when [^{3}H]dihydrostreptomycin was cross-linked to the 70S ribosome, the labeled streptomycin was distributed between the 30S and the 50S subunits in a ratio close to 1 to 1 and that the same 30S proteins were labeled in the free 30S subunit or in the 70S ribosome (P. Melançon and L. Brakier-Gingras, unpublished results). In this study, we also found that the pattern of labeling of the 50S proteins with [^{3}H]dihydrostreptomycin was identical, whether the 50S subunit was free or associated with the 30S subunit (data not shown).

Discussion

The results in this study show that streptomycin can be covalently attached to the 50S subunit of *E. coli* by using a bifunctional cross-linking agent. This is the first demonstration of a direct interaction between streptomycin and the 50S subunit and the first characterization of the components involved in this interaction. Girshovich et al. (1976) have observed that streptomycin could be linked to the 50S subunit when an aryl azide derivative of streptomycin is reacted with the 70S ribosome, but they did not investigate whether they could label the 50S subunit in the absence of the 30S subunit. Other workers (Delihas et al., 1975; Martinez et al., 1978; Tritton, 1978) have shown that streptomycin induces conformational changes in the 50S subunit when it binds to the ribosome, but they did not determine whether these conformational changes resulted from a direct interaction between streptomycin and the 50S subunit or were mediated through the interaction between the drug and the 30S subunit.

The cross-linking of streptomycin to the 50S subunit by phenyldiglyoxal is specific, since it was abolished under con-

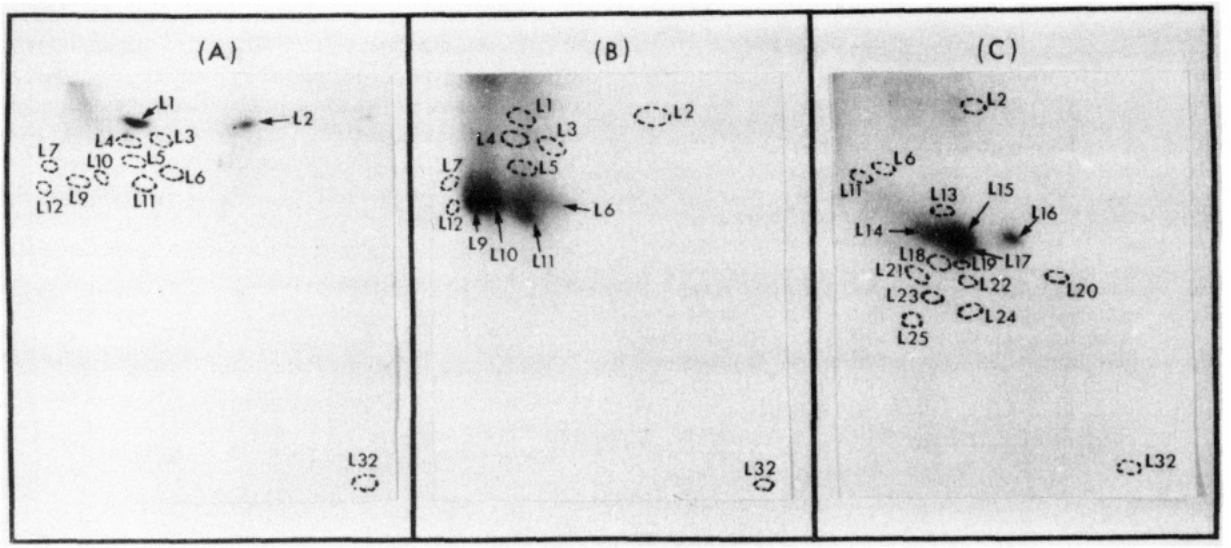

FIGURE 3: Identification by two-dimensional polyacrylamide/urea gel electrophoresis of the proteins present in the radioactive fractions indicated in Figure 2. Panels A, B, and C are autoradiograms which correspond to fractions I, II, and III of Figure 2, respectively. The autoradiograms were obtained 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 gels. Stained proteins are indicated by broken circles.

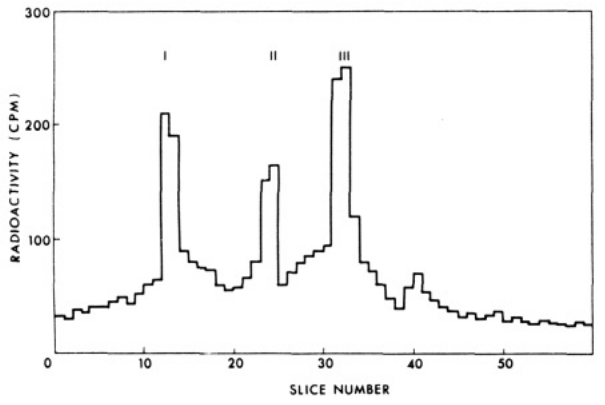

FIGURE 4: One-dimensional polyacrylamide/NaDodSO$_4$ gel electrophoresis analysis of 50S proteins from *E. coli* labeled with [^{3}H]-dihydrostreptomycin. Reported counts per minute are for protein extracted from 20 A_{260} units of 50S subunits.

ditions which prevent the binding of streptomycin to the ribosome. The cross-linking primarily involves the ribosomal RNA. However, we have focused here on the characterization of the proteins labeled with streptomycin, with the aim of determining the binding domain of streptomycin on the 50S subunit.

The 50S proteins labeled with streptomycin were L2, L6, and L17. These three proteins are near-neighbors since cross-links can be formed between L2 and L6, L2 and L17, and L6 and L17 (Traut et al., 1980). In addition, these three proteins are located at the interface between the 30S and the 50S subunits, since they can be cross-linked to 16S RNA (Sköld, 1981; Chiam & Wagner, 1983) and to proteins in the 30S subunit (Lambert & Traut, 1981; Cover et al., 1981). L2 and L6 can be cross-linked to the 30S proteins S11 and S13, respectively, and these latter two proteins were among those labeled with streptomycin, when the antibiotic was cross-linked to the 30S subunit with phenyldiglyoxal (Melançon et al., 1984). Proteins L2 and L17 can also be cross-linked to S12 and S4, respectively, and although these two 30S proteins were not labeled with streptomycin when phenyldiglyoxal was used, their involvement in the interaction of streptomycin with the 30S subunit has clearly been shown [Ozaki et al., 1969; Birge & Kurland, 1969, 1970; Hasenbank et al., 1973; reviewed in

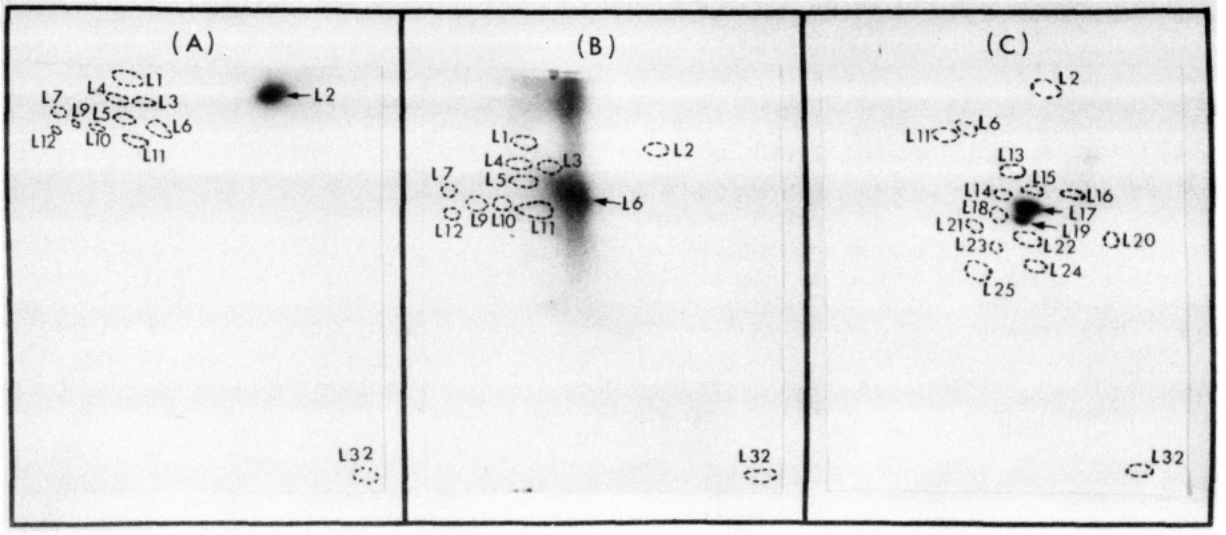

FIGURE 5: Identification by two-dimensional polyacrylamide/urea gel electrophoresis of the proteins present in the radioactive fractions indicated in Figure 4. See details in the legend to Figure 3 and under Materials and Methods. Panels A, B, and C are autoradiograms which correspond to fractions I,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

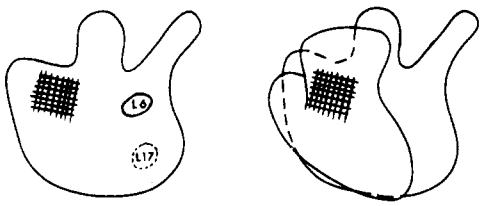

FIGURE 6: (A) 50S subunit model of Lake and Stöffler showing the internal surface of the subunit. (B) 70S ribosome, with the relative orientation of the subunits according to Lake (1982). The hatched areas represent the peptidyl transferase domain, which is in close proximity to the streptomycin binding site (see the text). The antigenic determinants for proteins L6 and L17, which are labeled with streptomycin, have been indicated. The dotted lines correspond to a localization for the antigenic determinant away from the viewer on the external face of the subunit while the solid lines correspond to a localization on the internal face of the subunit. The antigenic determinant for protein L2, which is also labeled with streptomycin, has not been mapped yet.

Wittmann & Wittmann-Liebold (1974)]. We conclude that the 50S proteins labeled with streptomycin delineate a single binding domain at the subunit interface, facing the binding domain of the drug on the 30S subunit. Furthermore, the same set of 30S and 50S proteins were labeled with streptomycin, whether the labeling was done with the free ribosomal subunits or with the 70S ribosome. All these data are consistent with the existence of a single binding site for streptomycin on the 70S ribosome (Chang & Flaks, 1972; Schreiner & Nierhaus, 1973; Grisê-Miron & Brakier-Gingras, 1982) which appears to be a composite site extending on both subunits.

Several approaches such as affinity labeling or reconstitution studies [reviewed in Cooperman (1980), Ofengand (1980), and Nierhaus (1982)] have indicated that proteins L2, L6, and L17 are neighbors to the peptidyl transferase domain, and we therefore suggest that the binding site for streptomycin on the 50S subunit is probably located close to that domain. Figure 6 relates our localization of the streptomycin binding site to the current models of the 50S subunit and the 70S ribosome, as determined by electron microscopy (Lake, 1980, 1982; Kastner et al., 1981; Stöffler-Meilicke et al., 1983a,b). The peptidyl transferase domain has been located in the valley between the central protuberance and the wider lateral protuberance (Lührmann et al., 1981; Olson et al., 1982). This domain faces the area on the 30S subunit where we have previously located the binding site of streptomycin (Melançon et al., 1984). The antigenic determinant for protein L6 has been mapped near the base of the thin lateral protuberance or stalk and that for L17, at the rear, on the external face of the subunit; the antigenic determinant for L2 has not yet been conclusively mapped (Lake & Strycharz, 1981; Stöffler-Meilicke et al., 1983a,b). Thus, in contrast to the evidence discussed above, L6 and L17 do not appear to be near-neighbors and to be close to the peptidyl transferase center, and L17 does not appear to be an interface protein. This apparent inconsistency can be explained by assuming that L6 and L17 are elongated proteins.

The translation initiation factor IF-3 can be cross-linked to the 30S subunit (Boileau et al., 1983; MacKeen et al., 1980) and also to the 50S subunit (Chaires et al., 1982; Schwartz et al., 1983). Proteins L2 and L17 are among the 50S proteins to which IF-3 can be bound. We have previously observed that there is some overlap between the binding site for streptomycin and that for IF-3 on the 30S subunit (Melançon et al., 1984). This study indicates that the binding site of streptomycin and that of IF-3 on the 50S subunit also overlap, since both these agents interact with proteins L2 and L17.

In conclusion, our results demonstrate that the target of streptomycin is not restricted to the 30S subunit but also extends to the 50S subunit. Since the functional centers of the ribosome are located in the area of contact between the 30S and the 50S subunits [reviewed in Cooperman (1980), Ofengand (1980), Cover et al. (1981), and Chiam & Wagner (1983)], the location of the streptomycin binding site in this important region can directly account for its interference with several steps of protein synthesis.

ACKNOWLEDGMENTS

We thank Dr. Guy Boileau, Gabriel Gingras, Muriel Herrington, and Michaël Laughrea for helpful discussions and comments.

Registry No. Streptomycin, 57-92-1; phenyl-1,4-diglyoxal, 53624-74-1.

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H_2 Histaminic Receptors in Rat Cerebral Cortex. 1. Binding of [^{3}H]Histamine†

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ABSTRACT: Saturable binding of [^{3}H]histamine in equilibrium with homogenates of rat cerebral cortex reveals Hill coefficients between 0.4 and 1.0, depending upon the conditions. Data from individual experiments are well described assuming one or two classes of sites. Only the sites of higher affinity (K_{P1} = 3.9 ± 0.5 nM) are observed when binding is measured by isotopic dilution at a low concentration of the radioligand (<1.5 nM) in the presence of magnesium or by varying the concentration of the radioligand. The sites of lower affinity (K_{P2} = 221 ± 26 nM) appear during isotopic dilution at higher concentrations of the radioligand or at lower concentrations either upon the addition of guanylyl imidodiphosphate (GMP-PNP) or upon the removal of magnesium. Estimates of the second- and first-order rate constants for association and dissociation of [^{3}H]histamine agree well with K_{P1}. Apparent capacities corresponding to K_{P1} and K_{P2} are of the order of 100 ($[R_1]_t$) and 1300 pmol/g of protein ($[R_2]_t$), respectively. Simple interconversion cannot account for the changes in binding that occur upon adding GMP-PNP or removing magnesium, since the increase in $[R_2]_t$ exceeds the decrease in $[R_1]_t$. Moreover, the apparent amount of high-affinity complex exhibits a biphasic dependence on the concentration of [^{3}H]histamine; an increase at low concentrations is offset by a decrease that occurs at higher concentrations. The latter appears to be positively cooperative and concomitant with formation of the low-affinity complex. These and other observations indicate that the binding of histamine is inconsistent with models commonly invoked to rationalize the binding of agonists to neurohumoral receptors. GMP-PNP and magnesium reciprocally alter capacity at the sites of higher affinity, however, and the reduction caused by GMP-PNP reflects a substantial increase in the rate constant for dissociation at the sites that appear to be lost. The sites labeled by [^{3}H]histamine thus reveal the properties of neurohumoral receptors linked to a nucleotide-specific G/F protein.

Saturable binding of [^{3}H]histamine first was reported by Palacios et al. (1978) in homogenates prepared from various regions of rat brain. Their observations subsequently were confirmed by ourselves (Kandel et al., 1980) and extended by Barbin et al. (1980). Apart from the report of Singh & McGeer (1979), there has been general agreement that the sites in mammalian brain bind histamine with an apparent dissociation constant of 7–9 nM and a capacity of the order of 100 pmol/g of protein. Comparisons of pharmacological specificity (Barbin et al., 1980; Kandel et al., 1980) and capacity (Kandel et al., 1980) have established that they are distinct from H_1 receptors labeled by [^{3}H]mepyramine (Hill et al., 1977; Tran et al., 1978) and from an imidazole-specific site labeled by [^{3}H]cimetidine (Burkard, 1978; Smith et al.,

† This investigation was supported by the J. P. Bickell Foundation and the Medical Research Council of Canada (Grants MT-3057 and MA-7130). J.W.W. is a Career Scientist of the Ontario Ministry of Health and during the course of this investigation was a Scholar of the Canadian Heart Foundation.

0006-2960/85/0424-6095$01.50/0