Ribosome Structure: Binding Site of Macrolides Studied by Photoaffinity Labeling†

Francisco Tejedor and Juan P. G. Ballesta*

Centro de Biología Molecular, CSIC and UAM, Canto Blanco, Madrid, Spain
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ABSTRACT: The macrolide antibiotics carbomycin A, niddamycin, and tylosin have been radioactively labeled by reducing their aldehyde group at the C-18 position. Dihydro derivatives with specific activities around 2.5 Ci/mmol can be obtained that, although partially affected in their activity, still bind to the ribosomes with high affinity. The presence in the chemical structure of these antibiotics of α–β-unsaturated ketone groups makes them photochemically reactive, and by irradiation above 300 nm, covalent incorporation of the radioactive dihydro derivatives into ribosomes has been achieved. The covalent binding seems to take place at the specific binding sites for macrolides as deduced from binding saturation studies and competition experiments with unmodified drugs. Analysis of the ribosomal components labeled by the drugs indicated that most radioactivity is associated with the proteins L27, L2, and L28 when 50S subunits are labeled, and with L27, L2, L32/33, S9, and S12 in the case of 70S ribosomes. These results agree well with a model of macrolides' mode of action that assumes an interaction of the drug at the peptidyl transferase P site that would block the exit channel for the growing peptide chain.

Thanks to the joint effort of a number of laboratories, a reasonably clear picture of ribosome structure is emerging. However, it is still far from explaining the different functional aspects of the activity of the particles (Wittmann, 1983; Liljas, 1982). Among other things, more data on the ribosomal components related to their activities are required in order to complete the localization of the different functional domains in the particle models.

Antibiotics have been a powerful tool in ribosomal studies. Identification of the ribosomal components involved in the binding process of the drugs has, in fact, allowed us to localize functional domains in the ribosome structure (Cundliffe, 1983). In these types of studies, affinity labeling is probably the technique that, under appropriate controls, has yielded more clear-cut results (Cooperman, 1980).

Among the macrolides, a generic name for compounds with a cyclic lactone structure, there are a number of antibiotics that specifically inhibit protein synthesis like erythromycin, spyramycin, carbomycin, etc. (Vázquez, 1979). Irrespective of their slightly different mode of action, all the macrolides tested compete for mutually exclusive sites on the ribosome (Vázquez, 1979). These sites must be localized near the peptidyl transferase, since macrolides inhibit the binding of their substrates and they also compete for binding to the ribosomes with typical peptide bond forming inhibitors such as chloramphenicol and lincomycin (Fernández-Muñoz et al., 1971). The indentification of the macrolide binding site might, therefore, provide interesting information on the structure of that important ribosomal active center.

There are a number of macrolide antibiotics that have in their structure potentially photoreactive chemical groups such as α–β-unsaturated ketones (Cowan & Drisko, 1978; Smith, 1970). Carbomycin A, niddamycin, and tylosin are among them, and since they can be made radioactive by reducing their aldehyde groups (Siegrist et al., 1981), they are good candidates with which to attempt the affinity labeling of their ribosomal binding site.

We report here the results obtained by this kind of approach that has allowed us to identify some ribosomal proteins involved in the macrolide binding center.

Materials and Methods

Preparation of Reduced Forms of Carbomycin (Pfizer), Niddamycin (Abbot), and Tylosin (Lilly Research Laboratories). Twenty-two micromoles of the antibiotics in 200 μL of diethylene glycol dimethyl ether was mixed with 110 μL of 2 mg/mL $NaBH_4$ (6 μmol) in the same solvent, and the reaction was allowed to proceed at room termperature for 12 h. Then, 0.5 mL of 0.2 M CO_3HNa, pH 9.35, was added and the mixture shaken for 15 min on an ice bath and extracted 3 times with 1 mL of Cl_2CH_2. The extract was checked by thin-layer chromatography (TLC)[1] on silica gel and eluted with Cl_3CH–CH_3OH (10:1). One major reaction product was detected and eluted from the plates.

When radioactive derivatives had to be prepared, the reduction was carried out with 50 mCi of NaB_3H_4 (220 mCi/mg) (Amersham International plc) and the reaction performed as above. The reaction products were detected by autoradiography of the TLC plates, and the one with an R_f identical with the major product in the nonradioactive reduction was eluted from the plate. The recovered compound was tested for purity by TLC.

Ribosomes. Ribosomes were prepared from *Escherichia coli* MRE 600 according to standard techniques (Staehelin & Maglott, 1971) and kept in buffer with 5 mM β-mercaptoethanol. When indicated in the text, ribosomes were dialyzed before used in order to remove the thiol.

Spectroscopic Techniques. UV–visible spectra of antibiotics were performed by using a Perkin-Elmer 554 double-beam spectrophotometer.

Infrared spectroscopy was carried out in KBr using Perkin-Elmer 325 equipment.

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[1] Abbreviations: TLC, thin-layer chromatography; PPO, 2,5-diphenyloxazole; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Cl_3CCOOH, trichloroacetic acid.

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NMR spectra were performed by Dr. M. Rico (Instituto Rocasolano, CSIC, Madrid) in a 90-mHz Bruker HX-90 spectrometer.

Electrophoretic Techniques. Ribosomal proteins and RNA were separated by urea–LiCl (Traub et al., 1971). When recovery of RNA was not required, ribosomes were treated with a mixture of RNase A and RNase T1 (Leon-Rivera et al., 1980).

The proteins were separated either by one-dimensional SDS gel electrophoresis (Weber & Osborn, 1969) or by standard two-dimensional electrophoresis (Kaltschmidt & Wittmann, 1972).

After electrophoresis, gels were stainded with 0.4% Coomassie blue in 50% ethanol 1–2 h in the absence of acetic acid and kept in 60% acetone for 2 h at 0 °C. Destaining was performed in 25% ethanol at room temperature overnight. The slabs were treated for fluorography with PPO in dimethyl sulfoxide (Bonner & Laskey, 1974), dried, and exposed at −70 °C.

Activity Tests. Synthesis of polyphenylalanine was performed in a *E. coli* system as described (Ballesta et al., 1974). Antibiotic binding to ribosomes was carried out either by filtration through nitrocellulose filters (Pestka, 1974) or by centrifugation through sucrose gradients. In this latter case, 0.1-mL reaction mixtures containing 0.3 μM ribosomes and 1 μM antibiotic in 60 mM NH_4Cl, 8–10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 4 mM β-mercaptoethanol were spun through 5-mL sucrose gradients (5–20%) in an SW 50.1 Beckman rotor for 1.5 h at 45 K.

Photoincorporation. Ribosome and antibiotic concentrations in the irradiated samples were 1–1.5 and 2.25 μM, respectively, in 80 mM KCl–10 mM $MgCl_2$. The solutions were buffered with either 10 mM PO_4^{3-} at pH 7.4 or 10 mM $BO_3H_2^-$ at pH 7.0 and 8.0. The samples were irradiated with a medium-pressure Hg lamp of 125 W, with a borosilicate glass filter, placed 5 cm from the tubes which were standing in a refrigerated bath to keep the temperature of the samples below 5 °C. The length of time of irradiation and the pH of the sample varied according to the experiment being performed as indicated in the text.

Results

Preparation of Reduced Forms of Carbomycin A, Niddamycin, and Tylosin. The antibiotics were treated with $NaBH_4$ under the conditions described under Materials and Methods and the products of the reaction separated by TLC on silica gel. One major reaction product was detected and eluted from the plates.

The reduced antibiotics were checked by UV–visible, infrared (IR), and nuclear magnetic resonance (NMR) spectroscopy. The results are in agreement with a specific reduction of the aldehyde group in position C-18 as expected (data not shown). Indeed, there is a clear diminution on the 1712 cm^{-1} band in the IR spectra, corresponding to the carbonyl group of both the lactone and the aldehyde at C-18. Also, the aldehyde proton signal at 9.6 ppm disappears from the NMR spectra. On the other hand, absorption at 240 nm for carbomycin A and at 280 nm for niddamycin and tylosin in the UV–visible spectra is still present in the reduced derivatives as is the ketone band at 1680 cm^{-1} in the IR scan, indicating that the carbonyl at position C-9 has not been affected.

Under the same conditions, but in the presence of tritiated $NaBH_4$, radioactive derivatives were also prepared. The spots moving in TLC to the position of the well-characterized nonradioactive reduced forms were recovered from the plates and used in the following experiments. Under the conditions of labeling used, specific activities in the range of 2.1–2.5 Ci/mmol were obtained for the reduced derivatives of the three antibiotics.

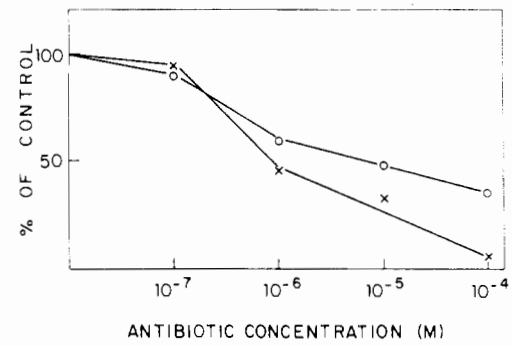

FIGURE 1: Inhibition of polyphenylalanine synthesis by carbomycin A (×) and dihydrocarbomycin A (○).

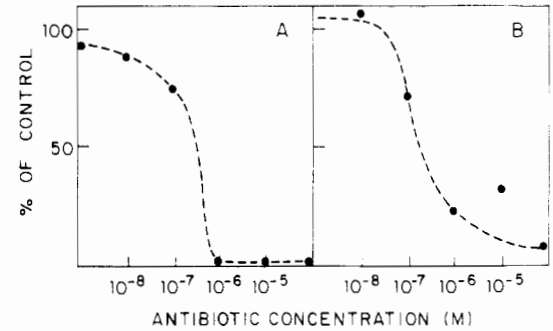

FIGURE 2: Competition of [^{3}H]dihydrocarbomycin A binding to the ribosomes by erythromycin (A) and carbomycin A (B). 70S ribosomes incubated in the presence of about 2-fold the concentration of dihydrocarbomycin A and of increasing concentrations of the competing drug were incubated as indicated under Materials and Methods and filtered through nitrocellulose filters. 100% binding correspond to 1800 cpm, equivalent to 0.34 molecule of drug per ribosome.

Biological Activities of the Reduced Macrolides. Reduction of the aldehyde at C-18 can affect the activity of macrolides (Siegrist et al., 1981). In our case, Figure 1 shows a partial inactivation of dihydrocarbomycin A when tested in a polyphenylalanine synthesizing system of *E. coli*.

On the other hand, the reduced form of carbomycin A binds with high affinity to the ribosomes of *E. coli*, and this binding is competed by both erythromycin and the unmodified antibiotic to the same extent (Figure 2).

When ribosomes incubated with radioactive antibiotics are partially dissociated by centrifugation through sucrose gradients, the radioactivity appears associated with the 50S and 70S particles but not with the 30S subunit (not shown), confirming the specificity of the binding.

Photoaffinity Labeling of Ribosomes. Ribosomes irradiated in the presence of radioactive drugs incorporate trichloroacetic acid precipitable radioactivity as a function of the time of irradiation (Figure 3). There are, however, clear differences in the amounts of radioactivity covalently incorporated by each antibiotic. While carbomycin A is extensively incorporated (as an average 3% of bound drug), niddamycin is much less reactive (only 0.5%) and tylosin practically unreactive.

Binding is not noticeably affected by changes in the pH of the reaction mixture, since at pH 7.0 and 8.0 (Figure 3) it is similar. However, it is very sensitive to the presence of SH-containing compounds in the sample. When ribosomes are not previously dialyzed to remove the β-mercaptoethanol, incorporation is reduced to approximately one-third that of the dialyzed controls (Figure 3).

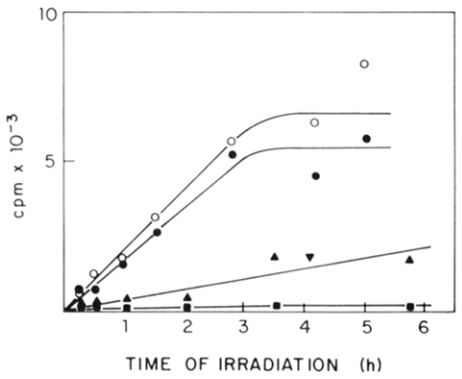

FIGURE 3: Covalent binding of dihydromacrolides to ribosomes. Ribosomes (1 μM), previously dialyzed to remove β-mercaptoethanol, were irradiated in 10 mM borate buffer, either pH 7.0 (closed symbols) or pH 8.0 (open symbols), in the presence of a 2–fold excess of [^{3}H]dihydrocarbomycin A (○, ●), dihydroniddamycin (▲), and dihydrotylosin (■). In the case of dihydrocarbomycin A, one sample (▼) was also included in which the ribosomes were not previously dialyzed, containing consequently a final concentration of 0.2 mM β-mercaptoethanol. Fifty-microliter samples were precipitated with 10% cold Cl_3CCOOH and filtered through glass fiber filters.

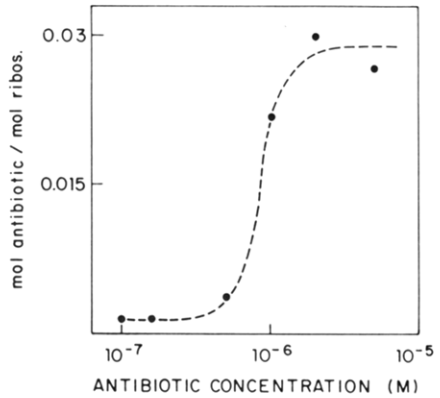

FIGURE 4: Covalent binding of dihydrocarbomycin A as a function of antibiotic concentration. Ribosomes (1.2 μM) in pH 7.4 phosphate buffer were irradiated for 3 h in the presence of the indicated [^{3}H]dihydrocarbomycin A concentrations. The samples were precipitated with 10% cold Cl_3CCOOH and filtered through glass fiber filters. Samples treated under the same conditions but kept in the dark were used as blanks, and their radioactivity was subtracted.

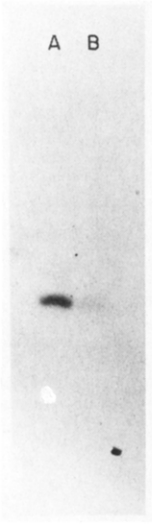

FIGURE 5: Autoradiogram by fluorography of SDS gel electrophoresis of proteins extracted from ribosomes irradiated in the presence of 2 μM [^{3}H]dihydrocarbomycin (A) and 2 μM [^{3}H]dihydrocarbomycin A with 10 μM carbomycin A (B). Irradiation was performed as in Figure 4.

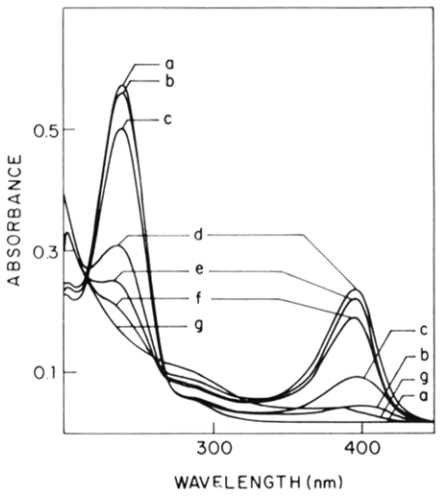

FIGURE 6: UV spectra of carbomycin A irradiated for 0 (a), 5 (b), 20 (c), 90 (d), 120 (e), 155 (f), and 280 min (g). Irradiation was performed in 10 mM borate, pH 7.0, 80 mM KCl, and 10 mM $MgCl_2$ with 5×10^{-5} M carbomycin.

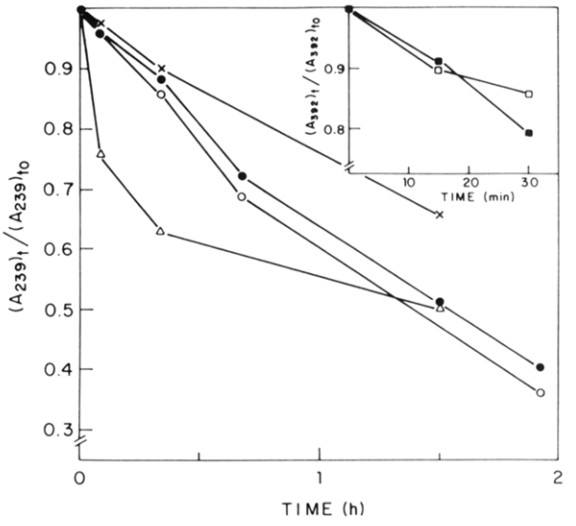

FIGURE 7: Degradation rate of carbomycin A and its photoproduct (inset). Carbomycin was irradiated alone (■, ●), under the conditions of Figure 6, and in the presence of 10^{-3} M lysine (△), 5×10^{-4} M cysteine (□, ○), and 2×10^{-3} M threonine (×). The relative concentrations of the compounds are given as the ratio of the absorption at the beginning of irradiation (A_0) to the absorption after a given time of irradiation (A_t). The maxima of absorption at 239 and 392 nm for carbomycin A and its photoproduct are taken from spectra in Figure 6.

Photoincorporation of antibiotics into the ribosome must occur at specific sites, since saturation is reached at a ratio of ribosome to antibiotics near 1 (Figure 4). The specificity of the covalent binding is confirmed in Figure 5, showing that unmodified carbomycin competes with the dihydro derivative for labeling the ribosomal proteins.

Mechanism of the Photoreaction. An attempt to understand the mechanism of the photoreaction that is taking place during irradiation of macrolides was performed by UV spectroscopy of carbomycin A photolysis. Upon irradiation of the antibiotic, there is a diminution of the drug absorption at 239 nm, with the simultaneous appearance of a compound absorbing at 392 nm which also disappears after extended irradiation of the sample (Figure 6).

To study the reactivity of macrolides and their photoproducts, irradiation experiments in the presence of different amino acids were performed. When the kinetics of the disappearance of the antibiotic were analyzed (Figure 7), it be-

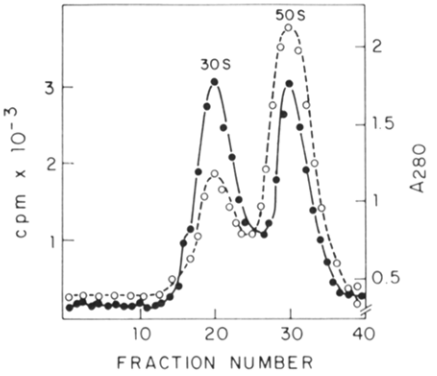

FIGURE 8: Distribution of covalently bound radioactivity between ribosomal subunits. Ribosomes irradiated under the conditions of Figure 4 in the presence of dihydrocarbomycin A were precipitated with 10% poly(ethylene glycol 6000) and collected by centrifugation. The pelleted ribosomes were resuspended in one-sixth of their original volume of 10 mM Tris-HCl, pH 7.4, 60 mM NH_4Cl, 1 mM $MgCl_2$, and 5 mM β-mercaptoethanol and separated by centrifugation through 5–20% sucrose gradients in the same buffer. Gradients were fractionated after centrifugation and fractions checked for A_{260} (○) and Cl_3CCOOH–precipitable radioactivity (●).

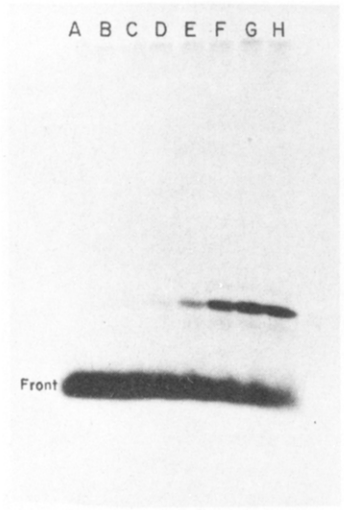

FIGURE 9: Autoradiogram by fluorography of SDS gel electrophoresis of ribosomes irradiated with dihydrocarbomycin A for different periods of time. Twenty-microliter aliquots of 1 μM ribosomes irradiated as in Figure 4 were made 1% SDS, incubated at 85 °C for 5 min, and directly applied to the gel. Samples were taken after irradiation for 0 (A), 10 (B), 30 (C), 60 (D), 90 (E), 180 (F), and 280 min (G). Radioactivity in the front corresponds to the unreacted drug.

came clear that cysteine accelerated the rate of degradation of the drug to a larger extent than lysine or threonine. On the other hand, the photoproduct was not affected by either cysteine (Figure 7) or lysine (not shown).

Identification of the Labeled Ribosomal Components. When ribosomes photolabeled in the presence of dihydrocarbomycin A are dissociated in sucrose gradients, radioactivity appears associated to 30S and 50S subparticles (Figure 8).

RNA and proteins of irradiated ribosomes were prepared, and 90% of the covalently bound radioactivity was found associated with the proteins. Proteins of ribosomes irradiated for different times in the presence of radioactive antibiotics were separated by SDS gel electrophoresis, and no qualitative change in the pattern of labeling as a function of the time of irradiation was found (Figure 9). Therefore, proteins of ribosomes irradiated for 4 h were separated by two-dimensional gel electrophoresis, and the radioactive spots were detected by fluorography. Fortunately, the spots appear in regions of the gel where, due to the isolation of the proteins, their identification is easy. As shown in Figure 10 and quantified in Table I, proteins L27, L2, and L28 in the 50S subunit are the major radioactively labeled components. In the 70S ribosome, proteins L27, L2, L32/33, S12, and S9 are labeled (Table I). It is interesting to note that protein L27 appears as a double-labeled spot, suggesting that more that one residue is susceptible to modification by the antibiotic.

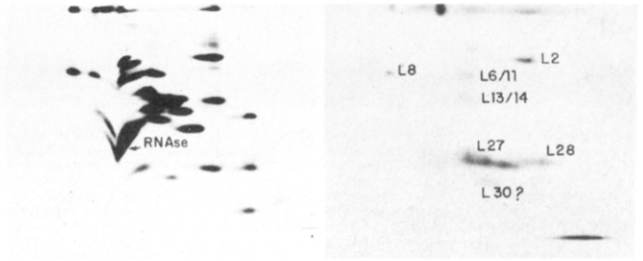

FIGURE 10: Two-dimensional gel electrophoresis of ribosomal proteins from 50S subunits irradiated in the presence of [^{3}H]dihydrocarbomycin A as in Figure 8: stained gel (left panel) and autoradiogram (right panel). The radioactive spot in the front does not correspond to any stained protein.

Table I: Estimation of Ribosomal Protein Labeling by Densitometry of Autoradiograms

protein	% total radioactivity in 50S particles	% total radioactivity in 70S particles
L2	11.5	5.4
L6/11	2.8	1.0
L8	2.9	1.1
L13/14	1.9	1.0
L27	65.8	48.6
L28	11.3	1.0
L30?	3.5	7.6
L32/33	1.0	5.3
S4		1.6
S9		5.2
S12		24.9

The minor spot below the position of protein L27 has not been totally identified. It is to the right of protein L30 while the other radioactive spots either coincide with or are to the left of their corresponding proteins.

Discussion

The reduction of the aldehyde group in carbomycin A, niddamycin, and tylosin yields radioactive dihydro derivatives of the drugs that bind to specific ribosomal sites with high affinity. Since they have in their molecules photoreactive α–β-unsaturated ketones, irradiation of dihydrocarbomycin A and dihydroniddamycin in the presence of ribosomes results in covalent incorporation of radioactivity into the particles. Dihydrotylosin, in spite of having similar reactive groups, is not photoincorporated.

The mechanism of the photoreaction responsible for the covalent incorporation of the antibiotics into the ribosomes is not totally understood. The fact that lysine has little affect on the photodegradation of carbomycin A together with the low sensitivity of the photoincorporation to the pH indicates that reaction with strong basic nucleophilic compounds like amino groups will not be very extensive. Cysteine, on the other hand, strongly reacts with the drug during photolysis, and this argues in favor of a photoaddition mechanism of SH groups to the double bond (Smith, 1970). In agreement with this possibility is the sensitivity of the photoreaction to the presence of β-mercaptoethanol.

However, additional mechanisms of reaction must be working, since β-mercaptoethanol does not inhibit totally the

photoincorporation and, as will be discussed later, proteins without cysteine in their structure are also labeled.

One possible source of errors in photoaffinity labeling experiments is the generation of photoproducts during the irradiation of the antibiotics. These compounds might be photoreactive and label other cellular components, though not at the specific binding site of the original effector (Goldman et al., 1983). In the case of the macrolides studied here, spectrophotometric analysis of the irradiated drugs clearly indicates the presence of one photoproduct that is also sensitive to irradiation. However, this photoproduct has a diferent reactivity than the original drug, and since its photodegradation is insensitive to the presence of cysteine and lysine, it probably did not interfere with the specific labeling reaction. In addition, it is known that the destruction of the unsaturated ketone in macrolides drastically affects their affinity for the ribosome (Siegrist et al., 1982). It is, therefore, improbable that at low concentrations significant binding of the photoproduct to the ribosomes takes place. Finally, the fact that when the time of irradiation is increased no qualitative changes are noted in the pattern of the labeled proteins in SDS gel electrophoresis indicates that, even in the improbable case of photoproduct reaction, the target is the same as that for the original drug. Therefore, the photoproduct incorporation would not alter the final results.

Different techniques and authors (Vázquez, 1979) have shown that the binding site for the macrolide antibiotic is in the large ribosomal subunit, although the small subunit must play its part, since mutations in this subunit affect the interaction of macrolides with the ribosome (Saltzman & Apirion, 1976; Pardo & Rosset, 1977). Our structural results confirm those functional data showing that the drugs are also photoincorporated into the small ribosomal subunit when experiments are performed with 70S particles. Unspecific binding cannot be totally excluded as possibly responsible for the small subunit labeling. We tend to believe, however, that as in other cases (puromycin, Nicholson et al., 1982a, b; chloramphenicol, Pongs & Messer, 1976; streptomycin, Leon-Rivera, 1981; tobramycin, Tangy et al., 1983; pactamycin, Tejedor et al., 1984) the binding must be located in the interface between both subunits. Therefore, although the components of the large subunit are probably directly responsible for the binding, the small subunit proteins are close enough to affect the binding, and also to be labeled as in our experiments.

Covalently incorporated radioactivity is mostly associated with proteins. In agreement with the involvement of SH groups in the photoincorporation reaction, several labeled proteins have cysteine in their structure (Moore, 1975). However, modification of others without cysteine, such as proteins L8, L32/33, and S9, argues in favor of alternative ways of covalent reaction. In this sense, it is also interesting to note that protein L27 (the most labeled protein) has only one cysteine residue (Chen et al., 1975) but presents at least two radioactive spots, indicating that, in addition, other amino acids must also be involved in the photoreaction. The bound antibiotic molecule might react with either one or the other residue, yielding in each case protein derivatives with different mobility.

Proteins L27 and L2 have been considered part of the peptidyl transferase center (Pellegrini & Cantor, 1977; Johnson & Cantor, 1980). The 30S proteins labeled in the experiments are close to them since S12 can be cross-linked to L2 and S9 to L2 and L27 (Cover et al., 1981). Proteins L6/11 have also been related to the peptidyl transferase activity (Pellagrini &

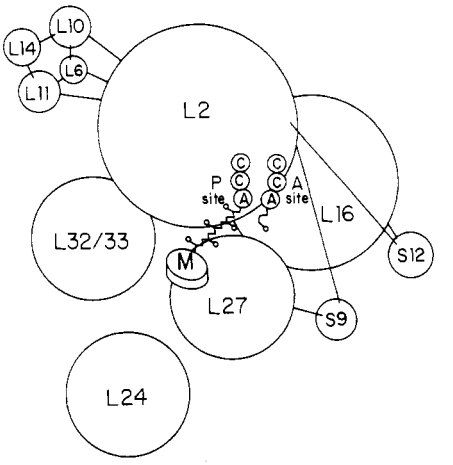

FIGURE 11: Model, after Eilat et al. (1974), of the possible arrangement of ribosomal proteins in the polypeptide chain exit channel in the 50S subunit. Proximities of other labeled proteins detected by cross-linking experiments (Traut et al., 1980) are also indicated. M indicates the macrolide molecule.

Cantor, 1977) and can be cross-linked to protein L2 (Traut et al., 1980). Finally, proteins L28 and L32/33 are in the interface region of the ribosome, and the last two can be cross-linked to S12 and S9 (Cover et al., 1981). It seems, therefore, that the labeled proteins form a cluster that must be localized in the region where protein L27 has been identified in the 50S ribosomal model (Lake & Strycharz, 1981). This region, on one side of the central protuberance closer to the lateral arm where protein L1 is located, is thought to be part of the peptidyl transferase center (Wittmann, 1983).

All these structural results agree well with the functional data in the literature indicating an interaction of these macrolides with the peptidyl transferase center of the ribosome (Vázquez, 1979). Although they inhibit binding of peptidyl transferase substrates to the ribosome, their capacity of inhibiting peptide bond formation in some model systems seems to be a function of the peptide chain size in the peptidyl-tRNA used as substrate, suggesting that a critical length is required for their inhibitory action (Contreras & Vázquez, 1977). These data indicate that macrolides probably block peptide bond elongation by steric hindrance of the growing peptide, when it reaches a critical length. A recent model has suggested that the final result of this block is the dissociation of the peptidyl-tRNA at the moment of the translocation from site A to site P (Menninger & Otto, 1982).

The functional data suggest, therefore, that the interaction of these macrolides with the ribosome probably takes place at the P site, near the exit channel for the growing polypeptide. If this is so, the proteins labeled in this study by the macrolides must form part of this ribosomal region. It is interesting to note that Eilat et al. (1974) have proposed a structure for the peptide exit region of transferase that fits in nicely with our results. According to their results, L2 would be part of the P site and the starting point for the channel, followed by L27 and L32/33. The macrolides, binding at protein L27, would block the peptide after three to six peptide bonds, as suggested by some functional data (Figure 11).

Finally, we have to bear in mind the fact that, although all the macrolides have a mutually exclusive binding site on the ribosome (Vázquez, 1979), clear differences can be seen in some aspects of their modes of action that make the existence of structural variation in the binding site possible.

In this sense, it is interesting to note that recently Le Goffic and co-workers (Sigriest et al., 1984) using rosaramycin have found labeling in proteins L1, L5, L6, and S1. These results,

although they can be due to the different labeling conditions used, might also reflect structural differences in the binding site of various macrolides.

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Registry No. Carbomycin A, 4564-87-8; dihydrocarbomycin A, 93894-87-2; [^{3}H]dihydrocarbomycin A, 93894-88-3; lysine, 56-87-1; threonine, 72-19-5; cysteine, 52-90-4; niddamycin, 20283-69-6; tylosin, 1401-69-0.

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