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Reaction of Some Macrolide Antibiotics with the Ribosome. Labeling of the Binding Site Components†

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ABSTRACT: Radioactive carbomycin A, niddamycin, tylosin, and spiramycin, but not erythromycin, can be covalently bound to *Escherichia coli* ribosomes by incubation at 37 °C. The incorporation of radioactivity into the particles is inhibited by SH– and activated double bond containing compounds but not by amino groups, suggesting that the reactions may take place by addition to the double bond present in the reactive antibiotics. This thermic reaction must be different from the photoreaction described for some of these macrolides [Tejedor, F., & Ballesta, J. P. G. (1985) *Biochemistry 24*, 467–472] since tylosin, which is not photoincorporated, is thermically bound to ribosomes. Most of the radioactivity is incorporated into the ribosomal proteins. Two-dimensional gel electrophoresis of proteins labeled by carbomycin A, niddamycin, and tylosin indicates that about 40% of the radioactivity is bound to protein L27; the rest is distributed among several other proteins such as L8, L2, and S12, to differing extents depending on the drug used. These results indicate, in accordance with previous data, that protein L27 plays an important role in the macrolide binding site, confirming that these drugs bind near the peptidyl transferase center of the ribosome.

The macrolides form a rather large group of natural compounds, having in their chemical structure a macrocyclic ring closed by lactonization, and showing inhibitory activity against living organisms (Hamilton-Miller, 1973). There are a number of antibiotics of great clinical importance among them (such as erythromycin, spiramycin, etc.) which specifically inhibit bacterial protein synthesis (Vázquez, 1979).

The macrolides which inhibit protein synthesis bind at mutually exclusive sites on the ribosome (Fernández-Muñoz et al., 1971). On the basis of their mode of action, they can be classified into two main groups represented by erythromycin

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and spiramycin, respectively (Vázquez, 1979), although recently a unifying model for the macrolides' mode of action has been proposed (Menninger & Otto, 1982). According to this model, macrolides bound to the ribosome interfere with the translocation of the peptidyl-tRNA from site A to site P, destabilizing the tRNA molecule and causing it to fall off the polysome.

These antibiotics have at least one carbonyl group in their structure. The carbonyl group is either an acetaldehyde or a ketone in the lactone ring. Many of them also have double bonds in the ring, in some cases conjugated with the carbonyl group of the ketone. These chemical characteristics make some macrolides potentially reactive, which led some investigators to test unsuccessfully the possible formation of covalent bonds in the interaction with their targets (Oleinick & Corcoran, 1969).

In fact, the potential reactivity of some macrolides has been useful for photoaffinity labeling studies that led to the identification of several components of the macrolide binding site (Siegrist et al., 1984; Tejedor & Ballesta, 1985).

During previous photolabeling studies, it was found that under certain conditions trichloroacetic acid resistant bonds could be formed between macrolides and ribosomes in the absence of irradiation. We have explored this fact in greater depth, and the results strongly suggest the existence of a thermic covalent reaction that was then used to perform affinity labeling studies of the ribosomal binding site of different macrolides. The results obtained are in accordance with those previously reported using photolabeling techniques (Tejedor & Ballesta, 1985).

Materials and Methods

Labeling of Macrolides. Radiolabeling derivatives of carbomycin, niddamycin, spiramycin, and tylosin were prepared through reduction of their aldehyde group with [^{3}H]borohydride as described previously (Tejedor & Ballesta, 1985). [*N-methyl*-^{14}C]Erythromycin was kindly supplied by Dr. R. E. McMahon (Lilly Research Laboratories, Indianapolis, IN).

Ribosomes were prepared from *Escherichia coli* MRE 600, grown in rich medium up to late exponential phase, according to standard procedures (Staehelin & Maglott, 1971). Particles were stored in 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] pH 7.4, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 5 mM mercaptoethanol.

Binding to Ribosomes. Total binding of labeled macrolides to ribosomes as well as competition experiments was performed as described previously (Tejedor & Ballesta, 1985).

Determination of Covalent Binding. Ribosomes (1.2 μM), extensively dialyzed against 10 mM borate, pH 7.0, 100 mM KCl, and 10 mM $MgCl_2$, were incubated with 0.5–1.0 μM [^{3}H]dihydro macrolide (2.5 Ci/mmol) or 0.1 mM [*N-methyl*-^{14}C]erythromycin (18 Ci/mol) in the dark. At the time indicated in the text, aliquots were taken and precipitated either with trichloroacetic acid or with acetone. In the first case, 1 mL of cold 10% trichloroacetic acid was added and the sample immediately filtered through fiberglass and washed with 10 mL of cold 5% trichloroacetic acid. In the second case, 3 volumes of acetone at −20 °C were added and the samples left at the same temperature for 3 h before being filtered through fiberglass filters, followed by washing with 10 mL of 66% acetone in 100 mM KCl at −20 °C. Nonincubated samples were used as blanks, and their radioactivity was subtracted from the results obtained after incubation.

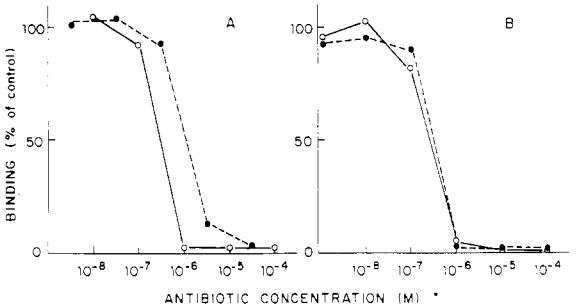

FIGURE 1: Competition of dihydroniddamycin (A) and dihydrotylosin (B) binding to ribosomes by erythromycin (●) and the respective unmodified drugs niddamycin (○) and tylosin (○). Ribosomes (0.2 μM) were incubated in the presence of twice the concentration of radioactive dihydro derivatives and of increasing concentrations of the competing drug. One hundred percent binding corresponds to 0.40 and 0.55 molecules per ribosome in (A) and (B), respectively.

Competition experiments were performed in similar conditions but with the addition of the required volume of the stock solutions of methyl vinyl ketone, 2-mercaptoethanol, and methylamine to obtain final concentrations of 0.02, 10, and 5 mM, respectively. Incorporation was stopped by adding to the incubation mixture, at the time indicated in the text, the required volume of 10 mM nonradioactive antibiotic to obtain a final concentration of 0.01 mM.

Photolabeling. Photolabeling of ribosomes using dihydro derivatives of carbomycin, niddamycin, and tylosin was performed by irradiation using wavelengths above 300 nm as described previously (Tejedor & Ballesta, 1985).

Electrophoresis of Labeled Ribosomal Proteins. Ribosomes incubated in the conditions described above were treated with a mixture of RNase A and RNase T1 as described previously (León Rivera et al., 1980) in order to degrade the rRNA. The proteins were precipitated in 3 volumes of acetone at −20 °C and separated in a scaled-down Kaltschmidt and Wittmann (1970) two-dimensional gel electrophoresis system loaded with 200–300 μg of protein. SDS gel electrophoresis was performed by standard methods (Weber & Osborn, 1969).

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

HPLC Chromatography of Labeled Proteins. Extracted proteins were dissolved in 15% acetonitrile in 0.1% trifluoroacetic acid and injected into a Ultrapore RPSC column (short alkyl chain, particle size 300 Å, pore size 5 μm, column size 75 × 4.5 mm) from Beckman. Elution was performed at room temperature at a constant flow rate of 0.5 mL/min using a 15–60% gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions were collected, dried on glass fiber filters, and counted.

Identification of the proteins in the eluted fractions was performed previously by two-dimensional gel electrophoresis, and the results (F. Polo, J. P. Abad, R. Amils, and J. P. G. Ballesta, unpublished results) coincide basically with previous reports (Kamp & Wittman-Liebold, 1984).

Results

Binding of Macrolides to Ribosomes. Reduced derivatives of macrolides lost from 20% to 50% of their inhibitory activity (Rakhit & Singh, 1974; Kirst et al., 1982) but were still able

[1] Abbreviations: PPO, 2,5-diphenyloxazole; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-pressure liquid chromatography.

Table I: Incorporation of Radioactivity into Ribosomes after Incubation with Radioactive Macrolides[a]

macrolide	incorporated radioactivity: total cpm/pmol of ribosomes	total pmol/pmol of ribosomes	trichloroacetic acid precipitable (cpm/pmol of ribosomes)	acetone precipitable (cpm/pmol of ribosomes)
experiment I				
[^{3}H]dihydrocarbomycin	731	0.43	33	25
[^{3}H]dihydroniddamycin	709	0.42	30	17
[^{3}H]dihydrospiramycin	878	0.52	5	28
[^{3}H]dihydrotylosin	960	0.57	9	35
experiment II				
[*methyl*-^{14}C]erythromycin	132	3.30	1	1

[a] Tests were performed in 50-μL samples containing 10 mM borate buffer, pH 7.0, 100 mM KCl, 10 mM $MgCl_2$, and 1.2 μM 70S ribosomes. The antibiotic concentration was 0.5 μM in experiment I and 0.1 mM in experiment II. Incubation was done in the dark at 32 °C for 1 h, and the samples were precipitated either with 10% or with 3 volumes of acetone at −20 °C for 3 h. Unincubated samples were used as blanks.

Table II: [^{3}H]Dihydrocarbomycin A Incorporation into Ribosomes as a Function of Reaction Temperature[a]

temp (°C)	cpm/pmol
0	−1.2
22	20.3
30	21.2
37	26.9

[a] Reactions were performed as described in Table I, incubating at the indicated temperatures.

to bind specifically to the ribosomes, and binding is competed for by erythromycin as shown in Figure 1 for niddamycin and tylosin and as was previously reported for carbomycin A (Tejedor & Ballesta, 1985).

When the radioactive drugs are incubated in the dark with *E. coli* ribosomes in the absence of 2-mercaptoethanol, trichloroacetic acid precipitable and acetone-precipitable radioactivity is found to be associated with the particles. Precipitated radioactivity accounts for 3–6% of the antibiotic originally bound to the particles which ranges from 0.4 to 0.8 molecule per ribosome (Table I). The difference in the precipitable radioactivity detected by either trichloroacetic acid or acetone in the case of tylosin and spiramycin indicates that the bond formed is highly sensitive to acid. We must note, however, that for all the macrolides tested, loss of radioactivity was detected when the precipitated samples were left standing in trichloroacetic acid even at 0 °C. This sensitivity to acid hydrolysis made it necessary to avoid any treatment with acid throughout the manipulations of the labeled samples described in later experiments.

A similar experiment was performed with radioactive erythromycin (Table I). The results clearly indicate that although the drug binds to the ribosomes, no covalent reaction is detected by using either trichloroacetic acid or acetone precipitation in spite of the fact that the drug concentration was 200-fold higher than in the experiments with the other macrolides. Table II shows that the reaction is temperature dependent and does not take place at 0 °C.

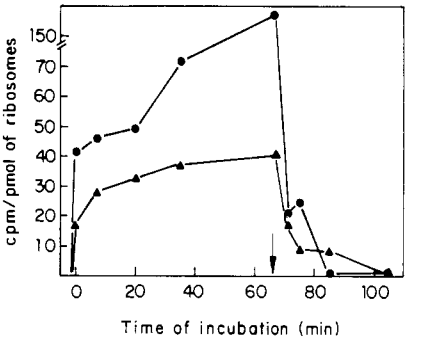

FIGURE 2: Kinetics of macrolide incorporation and reversibility of the reaction. 70S ribosomes of *E. coli* were incubated in the presence of 1 μM antibiotic as described under Materials and Methods. At the time indicated by the arrow, nonradioactive antibiotic was added up to 10 μM final concentration. 50-μL aliquots were removed and precipitated with acetone. Carbomycin (●); tylosin (▲).

Kinetics and Reversibility of Incorporation. The macrolide incorporation reaction is quite fast since, as shown in Figure 2, almost 50% of the incorporation takes place during the first minute of incubation. In the case of tylosin, the incorporation levels off after about 30 min of incubation. The kinetics of carbomycin incorporation, on the other hand, seem to be biphasic. After an initial rapid incorporation, a second slower process takes place. In both cases, however, the reaction is reversed by the addition of nonradioactive drug (Figure 2).

Competition Studies. To investigate the possible mechanisms of incorporation, competition experiments were performed in the presence of compounds carrying reactive groups that could be involved in the reaction, such as amino groups, SH groups, or activated double bonds. As shown in Table III, the reaction of niddamycin and tylosin is almost totally abolished by 2-mercaptoethanol and methyl vinyl ketone while the reaction of carbomycin A is inhibited only 50% by these compounds. On the other hand, amino groups are poor competitors in the reaction. We have previously tested that in-

Table III: Effect of Different Chemicals on Incorporation of Macrolides into Ribosomes[a]

	incorporation of radioact. (cpm/pmol) in: control	methyl vinyl ketone	2-mercaptoethanol	methylamine
experiment I				
[^{3}H]dihydrocarbomycin	32	17	23	nt
[^{3}H]dihydroniddamycin	16	0	0	nt
[^{3}H]dihydrotylosin	11	2	0.4	nt
experiment II				
[^{3}H]dihydrocarbomycin	67	nt	nt	57
[^{3}H]dihydrotylosin	45	nt	nt	43

[a] The tests were performed under the same conditions as described in Table I. The concentrations of methyl vinyl ketone, mercaptoethanol, and methylamine were 0.2, 10, and 5 mM, respectively; nt, not tested.

Table IV: Bound Radioactivity in Ribosomal Components after Incubation with Tritiated Macrolides[a]

macrolide	total radioact. (cpm) in rRNA	total radioact. (cpm) in proteins
[^{3}H]dihydrocarbomycin	55	1615
[^{3}H]dihydroniddamycin	168	1632
[^{3}H]dihydrotylosin	175	1775

[a] Ribosomes and antibiotics were incubated as described in Table I. Ribosomal proteins and RNA were separated by LiCl/urea.

cubation of the ribosomes with these chemicals does not substantially affect their activity (data not shown). The inhibition of the macrolide reaction in the presence of the competitor compound cannot be interpreted, therefore, as the result of disorganization of the ribosome structure.

Ribosomal Components Labeled in the Thermic Reaction. Ribosomes were made to react, as described under Materials and Methods, with the dihydro derivatives of carbomycin A, niddamycin, and tylosin. All the reactions and subsequent manipulations of the samples were carried out in the dark to prevent photoreactions. More than 90% of the radioactivity appears associated with the protein moiety of the ribosomes (Table IV) as is also the case in the photoincorporation of the macrolides (Tejedor & Ballesta, 1985). The specificity of the binding to the ribosomal proteins is confirmed, showing that the labeling is time dependent and is competed by the unlabeled antibiotic as well as by erythromycin (Figure 3).

With non-acid treatment, proteins were extracted from the particles and then resolved by two-dimensional gel electrophoresis and the radioactive spots detected by autoradiography [not shown; see Tejedor & Ballesta (1985)]. The spots in the autoradiogram were quantified by computerized densitometry and the results summarized in Table V. For comparison, results obtained by photoincorporation with carbomycin A (Tejedor & Ballesta, 1985) and niddamycin are also included in Table V. As previously indicated, tylosin is not photoincorporated by photoactivation in spite of having potential photoreactive groups, for reasons that are not well understood (Tejedor & Ballesta, 1985).

Identification of the proteins labeled in the 50S subunit was also carried out by reversed-phase HPLC. When dihydrocarbomycin A labeled proteins were analyzed by this technique, five radioactive peaks were detected in the chromatogram (Figure 4). Four of them coincide with identified ribosomal proteins, and one corresponds to a minor A_{214}-absorbing peak that was not detected in untreated samples. The major radioactive peak, accounting for about 45% of total radioactivity, corresponds to protein L27. About 8% of the

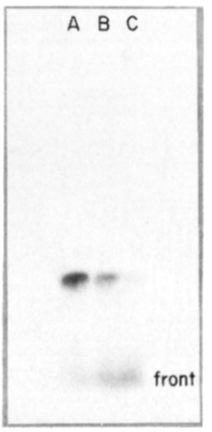

FIGURE 3: SDS gel electrophoresis autoradiogram of 50S ribosomes incubated with [^{3}H]dihydrotylosin. Samples incubated with 1 μM [^{3}H]dihydrotylosin (A), 1 μM [^{3}H]dihydrotylosin plus 1 μM unlabled tylosin (B), and 1 μM [^{3}H]dihydrotylosin plus 10 μM erythromycin (C) were made 1% in SDS, incubated at 85 °C for 5 min, and applied to the gel. Radioactivity in the front corresponds to the unreacted drug.

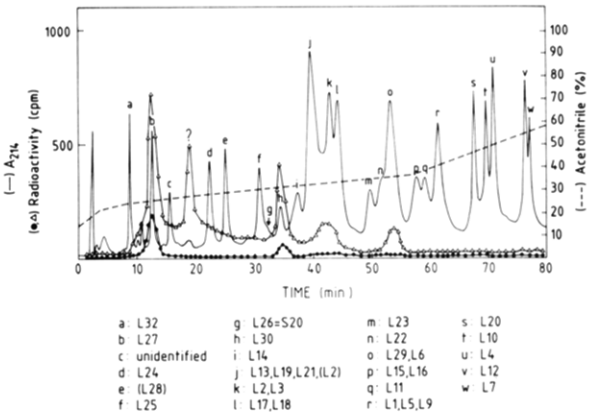

FIGURE 4: HPLC chromatogram of ribosomal proteins from the 50S subunit of *E. coli*. Identification of the proteins in the peaks was performed by two-dimensional gel electrophoresis (F. Polo, J. P. Abad, R. Amils, and J. P. G. Ballesta, unpublished results). Our identification basically agrees with the one by Kamp and Wittmann-Liebold (1984). Proteins extracted either from 2 mg of [^{3}H]carbomycin A labeled 50S subunits (▲) or from the region of the two-dimensional gel where the main radioactive spot is detected (Tejedor & Ballesta, 1985) (●) were dissolved in 15% acetonitrile in 0.1% trifluoroacetic acid and injected into the column. In the second case, unlabeled 50S subunit proteins (500 μg) were also injected as carrier. The shape of the acetonitrile gradient is indicated by the discontinuous line.

radioactivity is associated with a peak formed mainly by proteins L2 and L3, and it must correspond to the spot near protein L2 in two-dimensional gels. In the position of proteins

Table V: Quantitative Estimation of Ribosomal Protein Labeled by Photoactivated and Thermic Reactions of Macrolides[a]

protein	% radioactivity in proteins labeled by: carbomycin A, thermic	carbomycin A, photoactivated	niddamycin, thermic	niddamycin, photoactivated	tylosin, thermic
L2	7.3	5.4	7.6	7.5	5.0
L6	4.6	tr	13.2	16.3	12.4
L8	10.9	tr	12.3	13.3	16.4
L13/L14	tr	tr	5.6	4.9	3.6
L24/S13	4.8	0.0	3.6	tr	tr
L27	33.3	48.6	35.7	36.3	43.1
L30	13.0	7.6	tr	tr	68
L32/33	0	5.3	tr	tr	tr
S9	8.3	5.2	5.3	3.9	tr
S12	8.7	24.9	12.0	13.1	5.6

[a] Quantification of the spots in the autoradiograms was performed by using a Photomation P1700 densitometer from Optronics connected to a Pdp-11 Digital computer. Results are presented as a percent of the total radioactivity in the proteins. Amounts lower than 3% are listed as traces (tr).

L6 and L29, a peak with around 4% of the radioactivity is found, and since there is no spot in the two-dimensional gel near protein L29, it should correspond to protein L6. Finally, 20% of the radioactivity is associated with protein L30. This last result confirms the tentative identification of this protein in our previous report (Tejedor & Ballesta, 1975) and, in addition, indicates that its labeling is considerably higher than calculated from the two-dimensional electrophoresis results (Table V) probably because part of the labeled polypeptide does not enter the polyacrylamide gel.

We could not find radioactivity associated with proteins L10 and L7/12 in the chromatogram which could account for the spot found in protein L8 by two-dimensional electrophoresis, and therefore, definitive confirmation of the labeling in this protein requires additional data.

Identification of the 30S subunit proteins labeled in 70S ribosomes could not be carried out since the HPLC resolution of the proteins from the whole particle does not allow unambiguous conclusions. Therefore, the assignment of radioactivity to the small-subunit proteins in Table V has to be considered as tentative.

The assignment of the main radioactive spot in the two-dimensional electrophoresis gel to protein L27 was confirmed by cutting and eluting that part from unfixed and unstained slabs and analyzing the extract by HPLC. As shown in Figure 4, two radioactive peaks, corresponding to the positions of proteins L27 and L30, are detected.

Discussion

Our results clearly show that the macrolides tested, including carbomycin, niddamycin, tylosin, and spiramycin, are able to bind to ribosomal components through a reversible thermic reaction that probably results in the formation of stable bonds which resist trichloroacetic acid and/or acetone treatment.

The nature of the bond formed, as well as the reaction mechanisms, is not completely understood, and it is possible, at least in the case of carbomycin, that more than one reaction might take place. However, the sensitivity of the reaction to vinyl ketone and mercaptoethanol, as well as the low sensitivity to amino groups, is compatible with addition of SH-containing compounds to the double bond present in the lactone ring of all the macrolides tested. This agrees with results obtained from the study of the reaction of rosaramycin with 2-mercaptoethanol (Siegrist et al., 1982) and with the lack of reaction in the case of erythromycin that lacks the double bond. The presence in the carbomycin A molecule of an epoxy group that can be involved in other types of chemical reactions might explain the somewhat different behavior of this antibiotic in the competition experiment shown in Table IV.

Due to the low sensitivity of the reaction to exogenous amino groups, the incorporation through the formation of a Schiff base between the amino groups of proteins and the carbonyl group in the lactone ring of the drugs is improbable. This conclusion is also supported by the fact that our attempts to stabilize radioactive incorporation by reduction with borohydride failed (not shown). In addition, it is obvious that a Schiff base cannot be formed in the case of dihydrospiramycin since it lacks the carbonyl group. Whatever the reactions that take place are, the bonds formed resist all the extraction and electrophoresis procedures used for analysis of ribosomal components, and radioactivity can be detected in association with the proteins in the autoradiograms. This fact allowed us to use the thermic reaction for affinity labeling of the ribosomal proteins involved in the binding site.

Affinity labeling studies of the macrolide binding site had been performed previously using a photoactivated reaction in conditions that excluded thermic incorporation (Tejedor & Ballesta, 1985). We know very little about the mechanisms involved in either the photoactivated or the thermically activated reactions, but they must be different, at least in part, since tylosin, which cannot be photoincorporated (Tejedor & Ballesta, 1985), clearly participates in the thermic reaction. It is therefore interesting that the labeling results obtained with both types of reactions are similar. Therefore, our results make it less likely that the reactive groups in the antibiotics are labeling only a subset of the components of the binding site.

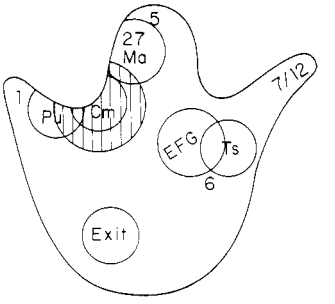

FIGURE 5: Model of the 50S ribosomal subunit. The positions of various proteins, indicated by their corresponding numbers, have been marked, along with several important sites such as the elongation factor EF G binding site (EFG), the growing polypeptide exit (Exit), and thiostrepton (Ts), puromycin (Pu), and chloramphenicol (Cm) binding sites. The hatched area corresponds to the possible location of the peptidyl transferase center.

The first thing that appears from the analysis of the results in Table V and Figure 4 is that protein L27, accounting for about 40% of the radioactivity, is the major labeled component in all cases regardless of the antibiotic and the type of reaction used. These data agree with the results previously reported (Tejedor & Ballesta, 1985), indicating that protein L27 is probably a very important part of the macrolide binding site. As previously discussed, L27 has been reported as a probable part of the peptidyl transferase P site (Eilat et al., 1974). On the basis of these data, a model has been proposed (Tejedor & Ballesta, 1985) summarizing these results and the results of others (Menninger & Otto, 1982; Contreras & Vázquez, 1976). This model proposes that macrolides block the movement of the nascent peptide, causing the release of the peptidyl-tRNA during translocation, after the formation of the first few peptide bonds.

Our results and previous results suggest that the macrolide binding site must be near protein L27. L27 has been located in the central protuberance of the large subunit, close to the chloramphenicol and puromycin binding sites (Lake & Strycharz, 1981). This region on the 50S particle is where the peptidyl transferase center is probably located [see Liljas (1982) and Wittmann (1983) for reviews on the peptidyl transferase center] (Figure 5).

Considerable radioactivity is associated with protein L30 when analysis is performed by HPLC. We had previously tentatively ascribed to this protein a radioactive spot moving near to its position and below the radioactivity assigned to protein L27 in two-dimensional electrophoresis (Tejedor & Ballesta, 1982). In fact, when that part of the gels is extracted, radioactivity appears associated with both proteins in HPLC (Figure 4).

Little is known about the structural and functional characteristics of this protein (Liljas, 1982), and therefore, additional data are required to clarify the significance of these results.

Radioactive macrolides are also linked to other proteins of the large subunit such as L2, L6, L24, L32/33, and L10 (as part of L8) to differing extents depending on the antibiotic

used and the type of reaction. All these proteins have been associated with the peptidyl transferase center by affinity labeling (Coopermann, 1980; Ofengand, 1980) and reconstitution techniques (Hampl et al., 1981) or have been cross-linked to proteins that are part of this active center (Kenny & Traut, 1979). However, the variation in results obtained by using different macrolides may not imply differences between the antibiotics' binding sites.

The labeling of some small-subunit proteins deserves comment. Although only tentatively identified, S12 and S9 seem to be labeled in every case and in some instances with relative intensity. We consider these results indicative of the proximity of these proteins to the binding site of the macrolides and therefore to the peptidyl transferase center. This was, in fact, previously suggested by the cross-linking of both proteins to peptidyl transferase components like proteins L2, L16, and L27 (Cover et al., 1981). Moreover, mutations in protein S12 affect the binding of macrolides to ribosomes from erythromycin-resistant strains (Saltzman & Apirion, 1976). The location of both the peptidyl transferase center and the binding sites of several other antibiotics at the subunit interface of the ribosome has also been suggested by affinity labeling studies using different types of probes that in most cases also label proteins from the small subunit (Nicholson et al., 1982a,b; Pongs & Messer, 1976; Tangy et al., 1983; Tejedor et al., 1985.)

On the basis of affinity labeling results using the macrolide rosaramycin, proteins L18 and L19 have been suggested as components of its binding site (Siegrist et al., 1985). Protein L18, as part of the complex with 5S RNA, has been located in the central protuberance of the 50S subunit (Stöffler-Meilicke et al., 1983) and therefore close to protein L27. Protein L19, on the other hand, seems to be one of the few proteins having immunogenic determinants in the bottom part of the large subunit (Stöffler-Meilicke et al., 1983), far away from the peptidyl transferase center (Wittmann, 1983), and although participation of this protein in the structure of this active center cannot be totally excluded, its labeling by rosaramycin was unexpected.

Proteins L4 and L22 seem to be involved in macrolide binding since they have been reported to be altered in erythromycin-resistant mutants (Tanaka et al., 1968; Wittmann et al., 1973); however, they are not labeled by any of the macrolides used so far. These results might indicate that the ribosomal binding sites for different macrolides, although overlapping, are not identical. Alternatively, proteins L4 and L22 may not form part of the macrolide binding sites, but they can affect its conformation by allosteric effects. A similar situation seems to occur in streptomycin binding to the ribosome. In this case, protein S12, altered in the best-characterized resistant mutants (Funatsu & Wittmann, 1972; Ozaki et al., 1969), has not been labeled by affinity labeling (Pongs & Erdman, 1973; Girshovich et al., 1976; Melançon et al., 1984).

We are presently carrying out affinity labeling experiments using different erythromycin derivatives, and our results indicate labeling of proteins L4 and L22, confirming their proximity to the erythromycin binding site (F. Tejedor, M. A. Arévalo, F. Polo, and J. P. G. Ballesta, unpublished results).

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Immunoelectron Microscopic Analysis of the Binding of Monoclonal Antibodies to Molecular Variants of Human Placental Alkaline Phosphatase†

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ABSTRACT: Three monoclonal antibodies with distinct antigenic specificities were examined by electron microscopy for their binding to three common genetic variants (SS, FS, and FF) of human placental alkaline phosphatase. In the reaction with the monoclonal antibody H5, all three variants of human placental alkaline phosphatase preferentially formed circular immune complexes composed of two antibodies and two enzyme molecules. In separate reactions with the F11 and B2 monoclonal antibodies, the SS variant formed circular complexes and the FS variant formed Y-shaped complexes composed of one antibody and two enzyme molecules, whereas the FF variant scarcely reacted. These results confirm immunochemical data showing that H5 binds to both S and F subunits with similar affinities, whereas F11 and B2 bind the S subunit with markedly higher affinity than they do the F subunit. Furthermore, the formation of circular complexes in the reaction of the mixture of the two antibodies, F11 and B2, with FS molecules suggests that these two antibodies bind to different sites on the S subunit. Therefore, the F and S subunits differ from one another at more than one site. This is the first indication that alleles of human placental alkaline phosphatase may result from more than just single point mutations in the gene encoding them.

Human placental alkaline phosphatase [orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1, PLAP[1]] is a membrane-bound enzyme composed of two subunits with molecular weights of approximately 65 000 each (Badger & Sussman, 1976; Holmgren & Stigbrand, 1976). In an electron microscopic study using negative staining, the native molecule appeared rectangular in shape with a longitudinal stain-filled groove and with each of the half-molecules (presumably each of the subunits) very often appearing to be bilobed (Takeya et al., 1984).

PLAP is a highly polymorphic protein with many more genetic variants than any other enzyme studied to date (Harris, 1980). The enzyme is coded by 3 common alleles and at least 15 rare alleles at a single autosomal locus. The most common alleles are pl_1 and pl_2 with gene frequencies of 0.62 and 0.28 among Caucasians, although these frequencies vary considerably in other population groups (Donald & Robson, 1974). Since the enzyme is a dimer, these two alleles give rise to three phenotypes expressed as SS, FS, and FF (new nomenclature 1, 2-1, and 2). Although these phenotypes are easily distinguishable on starch gel electrophoresis, the structural basis for the electrophoretic differences between the products of different alleles has not been elucidated. SS and FF types of PLAP have similar biochemical and physicochemical properties (Holmgren & Stigbrand, 1976). In an electron microscopic study, no significant difference in shape and size was observed between these two molecules (Takeya et al., 1984). They can be distinguished immunochemically if exstensive cross-absorption of polyclonal antisera is performed (Wei & Doellgast, 1980) or monoclonal antibodies (mAbs) are used (Slaughter et al., 1981; Millán et al., 1982; Jemmerson et al., 1985).

In the present study, we employed mAbs with distinct antigenic specificities for PLAP to analyze their interaction with different variants of PLAP by high-resolution electron microscopy. Immune complexes of three mAbs (H5, F11, and B2) and three types of PLAP molecules (SS, FS, and FF) were examined. The purposes of this investigation were, first, to visualize and analyze the immune complexes in the electron microscope and, second, to observe relative locations of the antigenic differences between the common allelic variants of PLAP.

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[1] Abbreviations: PLAP, human placental alkaline phosphatase; mAb, monoclonal antibody; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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