Binding of *N*-Substituted Erythromyclamines to Ribosomes

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Several *N*-substituted erythromyclamines were evaluated for their ability to inhibit the binding of [¹⁴C]erythromycin to ribosomes. The association and dissociation constants for the binding of each compound to *Escherichia coli* ribosomes were determined. These studies have resulted in the development of three types of probes for topological studies of the erythromycin-binding site and the ribosome: the chemically reactive bromoacetamido, the photoreactive *N*-(2-nitro-4-azidophenyl)glycinamido, and the fluorescent fluorescein isothiocyanate derivatives of 9(S)-erythromyclamine.

Erythromycin, a macrolide antibiotic, inhibits protein synthesis as a consequence of its ability to bind tightly to the 50S subunit of bacterial ribosomes (5, 8, 13). In a previous investigation, several erythromycin analogues were synthesized to determine which areas of the erythromycin molecule could be modified with retention of ability to bind to ribosomes (10). These studies indicated that the 9-position of the erythromycin molecule (Fig. 1) should readily accommodate reactive groups with radioactive labels for affinity labeling of ribosomal components. In addition, it was anticipated that the erythromycin molecule should be able to carry a number of fluorescent probes into the erythromycin ribosomal-binding site. Such probes could be used for studying the three-dimensional structure of ribosomes by various fluorescence techniques (2). By combining these techniques, it should be possible to develop a topological map of the erythromycin-binding site and adjacent areas.

With the above concepts in mind, various *N*-substituted erythromyclamines were prepared in which the 9-position was substituted with either a chemically reactive, photosensitive, or fluorescent group. The dissociation constants of the above erythromycin derivatives were determined by measuring their effects on [¹⁴C]erythromycin binding to ribosomes (3, 10).

EXPERIMENTAL PROCEDURES

The 9(S)- and 9(R)-erythromyclamines were obtained from Koert Gerzon (Lilly Research Laboratories) and Ronald LeMahieu (Hoffmann-La Roche, Inc.) and were prepared as described previously (6, 7). The *N*-substituted derivatives 48 to 52 shown

in Fig. 1 were prepared by condensation of erythromyclamine with the appropriate carboxylic acid using dicyclohexylcarbodiimide and *N*-hydroxysuccinimide by conventional methods (15). Thus, in a typical experiment 50 μ mol of dicyclohexylcarbodiimide in 1.5 ml of dimethylformamide was added to a mixture of 50 μ mol of the appropriately substituted carboxylic acid and 50 μ mol of *N*-hydroxysuccinimide in 2.0 ml of dimethylformamide. The reaction mixture was stirred at ambient temperature for 4 h, and 50 μ mol of 9(S)- or 9(R)-erythromyclamine in 0.5 ml of dimethylformamide was added. Formation of product was followed by thin-layer chromatography on silica gel 60 F-254 plates. The plates were developed with methylene dichloride, methanol, and ammonium hydroxide (90:10:1), sprayed with 5% phosphomolybdic acid in ethanol, and heated to 140 C. The erythromyclamine and the corresponding *N*-substituted derivative appeared as black spots. All these products exhibited a higher *R_f* than the parent amine in this chromatographic system. The compounds were purified by preparative thin-layer chromatography on glass plates (20 by 20 cm) coated with 1 mm of silica gel 60 F-254 developed with the above solvent system. All compounds were chromatographically homogeneous on thin-layer chromatography. Infrared spectra of compounds 48 to 52 were consistent with their respective molecular structures and exhibited amide bands at 1,650 and 1,550 cm^{-1} as expected.

The fluorescent derivative, 53, was prepared by allowing 9(S)-erythromyclamine to react with 1.25 equivalents of 5-fluorescein isothiocyanate (Eastman Kodak Co.) in acetone-water (1:1) containing two equivalents of potassium carbonate. The mixture was allowed to stand at room temperature overnight, and the solvents were removed by evaporation in vacuo. The yellow-orange residue was applied to a silica gel column, and the excess fluorescein isothiocyanate was eluted with ethyl acetate-acetic acid (9:1). The fluorescent product, 53, was then eluted with methanol. Evaporation of the methanol gave chromatographically pure 53 as a yellow-orange solid. Thin-layer chromatography on silica gel plates developed with methanol gave a single

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FIG. 1. Structure of erythromycin A and N-substituted erythromycylamines.

fluorescent spot (R_f 0.71). The spot blackened upon treatment with 5% phosphomolybdic acid. No erythromycylamine (R_f 0.07) was detected.

The numbering of the analogues in Fig. 1 is a continuation of the 45 previously tested erythromycin derivatives described in preceding papers (10, 11). Stock solutions of 0.01 M were prepared by dissolving the compounds in dimethylformamide or dimethyl sulfoxide. Dilutions were made directly into water. The erythromycin-binding assay with [¹⁴C]-erythromycin (45.7 mCi/mmol) and *Escherichia coli* ribosomes was performed as previously described (9). Analogue 48 (154 mCi/mmol) was prepared from

[³H]bromoacetic acid obtained from Amersham/Searle. Analogue 49 (940 mCi/mmol) was prepared by condensing [³H]N-(2-nitro-4-azidophenyl)glycine with 9(S)-erythromycylamine as described above. Both radiolabeled analogues 48 and 49 were chromatographically identical to the corresponding unlabeled materials, which were prepared in larger amounts and extensively purified. All chemical and biological studies with the photosensitive arylazides were performed in the dark with a safelight lamp equipped with filter no. 1 (Eastman Kodak) as a light source.

RESULTS AND DISCUSSION

The effects of antibiotics on [¹⁴C]erythromycin A binding to ribosomes are illustrated in Fig. 2. By replotting of the data of Fig. 2 by the method of Harris and Pestka (3), it was possible to compute the association (K_i) and dissociation (K_d) constants as well as the number of sites (n) on the ribosomes for each analogue. In Fig. 3, the corresponding plots for each of the N-substituted erythromycylamines are illustrated; and in Table 1, K_i , K_d , pK_d , and n are given for each analogue.

Examination of Table 1 reveals that the parent 9(S)- and 9(R)-erythromycylamine isomers 46 and 47 (Fig. 1) have K_i values 1.12 and 3.55 times greater than erythromycin A, respectively. The high binding affinities exhibited by the erythromycylamines suggested that the corresponding N-substituted derivatives would serve as potential candidates for affinity labeling and for fluorescent probes, which would be directed to the erythromycin-binding site. Thus, the corresponding photosensitive N-(2-nitro-4-azidophenyl)glycyl derivatives 49 and 50 were prepared to evaluate the accommodation of the arylazide by the ribosomal-binding site. The arylazide was selected because it is chemically stable at 37°C and can be photolyzed by visible radiation. The active product, an aryl nitrene (4), has an extremely broad spectrum of reactivity. For example, the nitrene can insert into almost any C-H bond and subsequently form a covalent link with the ribosome. Interestingly, the N-substituted amines exhibited a reversal of relative binding activities compared to the epimers of the parent amines. For example, the ratio of association constants of the 9(S) and 9(R) isomers 46 and 47 was 1:3.2; and for the respective glycylyl azides 49 and 50, the ratio of 9(S) to 9(R) isomers was 4.5:1 (Table 1).

Since the 9(S)-erythromycylamine yielded N-substituted derivatives with greater ribosomal-binding affinities, the 9(S) isomers were selected for our extended studies. A third photosensitive compound, the N-(2-nitro-4-azidophenyl)- γ -butyryl derivative, 51, exhibited an association

FIG. 2. Effect of erythromycin analogues on [^{14}C]erythromycin binding to ribosomes. Each 0.50 ml of reaction mixture contained the following components: 0.01 M KCl; 0.01 M NH_4Cl ; 0.004 M MgCl_2 ; 0.01 M tris(hydroxymethyl)aminomethane-chloride, pH 7.2; 1.1 μM [^{14}C]erythromycin; 7.5 absorbancy units at 260 nm of NH_4Cl -washed ribosomes; and erythromycin analogues at the concentrations indicated on the abscissas. Reactions were started by the addition of ribosomes and incubated at 24 C for 15 min. Assays were performed as previously described (9, 10). The data are presented as a percentage of the [^{14}C]erythromycin bound to ribosomes in the absence of any nonradioactive erythromycin or erythromycin derivatives.

constant 2.5-fold greater than that of 49. Since 51 differs from 49 by two methylene carbons, possible explanations for the increased binding of 51 may include additional hydrophobic interactions with the ribosome. Alternatively, the slightly longer chain length may relieve possible repelling interactions between the arylazide moiety and the surface of the ribosome.

The bromoacetamido derivative 48 and the chlorambucil derivative 52 represent two chemically reactive potential affinity labels. The use of chemically reactive moieties in active site-directed irreversible binding has been reviewed by Baker (1) and by Pellegrini and Cantor (*In H. Weissbach and S. Pestka, Protein biosynthesis*, in press). Examination of Fig. 2 and 3 reveals that both 48 and 52 inhibit [^{14}C]erythromycin binding to ribosomes. The K_d values (Table 1) demonstrate that both compounds show excellent potential for affinity binding studies. Although the chlorambucil derivative 52 yielded a higher association constant, the

greater stability of 48 and the ease of incorporating a radioactive label make the bromoacetamide derivative a more desirable candidate for structural studies of the erythromycin-binding site.

Since the binding studies with the above compounds indicate that the ribosome could tolerate fairly large substituents at the 9-position of the erythromycin molecule, it was anticipated that the bulky fluorescein isothiocyanate fluorescent probe would also be accommodated. Thus, 53 was prepared and tested for reversible binding to the ribosome. The K_d value (Table 1) indicates that this fluorescent derivative has excellent ribosomal-binding activity. The fact that the fluorescein isothiocyanate moiety represents a most satisfactory acceptor in energy transfer studies in ribosomal systems (2) makes 53 an excellent fluorescent marker for the erythromycin-binding site.

As predicted from previous studies on erythromycin analogues (10), the present data

FIG. 3. Graph of Y as a function of antibiotic concentration for computation of association and dissociation constants. The equation

$$\log \left[\left(\frac{K_0 [A] ([R_0] - [RA])}{[RA]} \right) - 1 \right] = n \log [I] + \log K_1 = Y$$

as derived by Harris and Pestka (3) was used to calculate K_1 , the association constant for the binding of inhibitor to ribosomes; K_0 is the association constant for the binding of [14 C]erythromycin to ribosomes ($9.9 \times 10^7 \text{ M}^{-1}$ was used for these calculations, reference 9); n is the number of inhibitor molecules binding to ribosomes; $[A]$ is the free [14 C]erythromycin concentration; $[R_0]$ is the total concentration of ribosomes potentially active in binding [14 C]erythromycin; and $[RA]$ is the concentration of the [14 C]erythromycin-ribosome complex. From the data in Fig. 2, Y was plotted as a function of $\log [I]$.

TABLE 1. Association constants (K_1), dissociation constants (K_d), and interaction coefficients for erythromycin analogues

Erythromycin analogue	$K_1 (\text{M}^{-1})$	$K_d (\text{M})$	$\text{p}K_d$	n
Erythromycin A	1.00×10^8	1.00×10^{-8}	8.00	1.02
46	1.12×10^8	8.90×10^{-9}	8.05	1.33
47	3.55×10^8	2.82×10^{-9}	8.55	1.11
48	8.93×10^6	1.12×10^{-7}	6.95	1.12
49	2.82×10^7	3.55×10^{-8}	7.45	1.05
50	6.33×10^6	1.58×10^{-7}	6.80	0.93
51	7.09×10^7	1.41×10^{-8}	7.85	1.11
52	7.94×10^7	1.26×10^{-8}	7.90	1.03
53	2.24×10^6	4.47×10^{-7}	6.34	1.04

demonstrate that the ribosome provides a suitable tolerance for relatively large structural moieties extended from the 9-position of eryth-

romycin A. In addition, the present results indicate that conversion of the sp^2 -hybridized C9 to the sp^3 -hybridized 9-erythromycylamine epimers does not diminish the binding activity of the erythromycin structure. Thus, three classes of N -substituted erythromycylamines were prepared and evaluated as potential probes of topological studies on ribosomes. In the photosensitive series, the 9(S) epimer [^3H]- N -(2-nitro-4-azidophenyl)glycyl derivative (49) was selected for photoaffinity labeling studies because of its greater binding affinity over the 9(R) epimer (50). Although the N -(2-nitro-4-azidophenyl)- γ -butyryl derivative (51) was slightly more active than 49, the synthetic difficulties encountered in incorporation of a radioactive label into 51 made it a less desirable analogue. For reasons explained above, the 9(S)-[^3H]bromoacetamide derivative, 48, was selected for irreversible binding studies in the chemically reactive series. Preliminary studies

have demonstrated that both 48 and 49 form covalent bonds with ribosomes. Studies are presently underway with these derivatives to localize the erythromycin-binding site of *E. coli* ribosomes (A. Zamir, M. Shapira, R. Vince, and S. Pestka, to be published). Also, recent studies have indicated that the fluorescent derivative, 53, provides an excellent probe for measuring the distance between the erythromycin-binding site and ribosomal proteins which are covalently conjugated with fluorescent donors (R. Langlois, C. C. Lee, C. Cantor, R. Vince, and S. Pestka, in preparation). Furthermore, the absolute rate constants for the forward and reverse reactions were calculated directly for compound 53 by fluorescence measurements. The dissociation constant, determined by the ratio of the rate constants, of 53 with 70S ribosomes was found to be 4.33×10^{-7} M. The dissociation constant determined by competition with [14 C]erythromycin binding to 70S ribosomes is 4.47×10^{-7} M (Table 1). The agreement by these independent measurements supports the validity of the competition assay for determination of binding constants and additionally suggests that compound 53 is not significantly contaminated by other compounds.

Of additional interest is the observation that the 9(S) epimer of erythromyclamine binds to ribosomes with an affinity 3.2-fold that of the 9(R) epimer. In contrast, however, the association constants of the glycyarylazides 49 and 50 were reversed, with the 9(S) epimer binding 4.5-fold better than the 9(R) derivative. Since the unsubstituted 9(R)-erythromyclamine binds to ribosomes better than the unsubstituted 9(S) epimer, it would have been anticipated that it would be a more effective antimicrobial agent (11, 12). Since this was not observed and, in fact, the 9(S) epimer was reported to be a significantly more effective antimicrobial agent than the 9(R) epimer (6), it is evident that additional factors account for the decreased antimicrobial activity of the 9(R) epimer. In general, it can be expected that the ability to bind to ribosomes should correlate with antimicrobial activity (11, 12). The low antimicrobial activity of the 9(R) epimer may thus be due to decreased bacterial permeability or to cellular inactivation of the 9(R) epimer. Since the 9(S) epimer (46) has an association constant equal to 1.12 that of erythromycin A, it would have been expected that its antimicrobial activity would approximate that of erythromycin A. In fact, however, its antimicrobial activity was about one-third that of erythromycin A (6). Thus, relative to erythromycin A, its

antimicrobial activity was significantly less than would be expected from studies of ribosomal binding. Therefore, we suggest that both 9(S)- and 9(R)-erythromyclamines are altered by bacteria, probably by modification of the 9-amino group. Since substitution of the 9(R) epimer radically inactivates binding activity, this could account for the poor activity of this derivative with intact cells. Furthermore, the observation that the 9(R) epimer (47) is effectively inactivated by substitution (50) suggests that the 9(R) amino group interacts with and points toward the ribosome in such a configuration that further substitutions interfere with ribosomal binding. In contrast, these results suggest that the amino group of the 9(S) epimer is directed away from the ribosome.

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