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Tight Binding of Clarithromycin, Its 14-(*R*)-Hydroxy Metabolite, and Erythromycin to *Helicobacter pylori* Ribosomes

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Clarithromycin is a recently approved macrolide with improved pharmacokinetics, antibacterial activity, and efficacy in treating bacterial infections including those caused by *Helicobacter pylori*, an agent implicated in various forms of gastric disease. We successfully isolated ribosomes from *H. pylori* and present the results of a study of their interaction with macrolides. Kinetic data were obtained by using ^{14}C-labeled macrolides to probe the ribosomal binding site. Clarithromycin, its parent compound erythromycin, and its 14-(*R*)-hydroxy metabolite all bound tightly to *H. pylori* ribosomes. K_d values were in the range of 2×10^{-10} M, which is the tightest binding interaction observed to date for a macrolide-ribosome complex. This tight binding was due to very slow dissociation rate constants of 7.07×10^{-4}, 6.83×10^{-4}, and 16.6×10^{-4} min^{-1} for clarithromycin, erythromycin, and 14-hydroxyclarithromycin, respectively, giving half-times of dissociation ranging from 7 to 16 h, the slowest yet measured for a macrolide-ribosome complex. These dissociation rate constants are 2 orders of magnitude slower than the dissociation rate constants of macrolides from other gram-negative ribosomes. [^{14}C]clarithromycin was bound stoichiometrically to 50S ribosomal subunits following incubation with 70S ribosomes and subsequent separation of the 30S and 50S subunits by sucrose density gradient centrifugation. These data predict that the lower MIC of clarithromycin compared with that of erythromycin for *H. pylori* is likely due to a faster rate of intracellular accumulation, possibly because of increased hydrophobicity.

The macrolide class of antimicrobial agents is over 30 years old and is still at the forefront of antimicrobial therapy as well as drug discovery and development (12, 15). Clarithromycin is a recently approved 14-membered macrolide with increased stability in acid and improved pharmacokinetics, including the appearance of a microbiologically active metabolite in humans (8). Clarithromycin possesses broad-spectrum antimicrobial activity, inhibiting a range of gram-positive and gram-negative organisms, some anaerobes, and atypical pathogens (1, 3, 14, 16, 25), in many cases with greater in vitro activity than erythromycin.

The importance of *Helicobacter pylori* to the field of gastroenterology has increased because of its potential etiologic role in disorders of the upper gastrointestinal tract (2, 6, 7, 26, 27). *H. pylori* with concurrent gastritis is common in patients with peptic ulcers, indicating a causal relationship to ulcer disease, and possible links may also exist between *H. pylori* and gastritis in the pathogenesis of gastric cancer (26). The treatment of gastric disease associated with *H. pylori* requires determination of the best therapeutic regimen by using proton pump inhibitors and antibiotics. The in vitro efficacy of clarithromycin against *H. pylori* (13, 17, 21) suggested its possible efficacy in vivo, and the results of initial clinical trials have been positive (4, 11, 24).

Little is known, however, regarding the interaction of macrolides with *H. pylori* ribosomes. In this communication we report the successful isolation of ribosomes from *H. pylori* and describe the kinetics of their interaction with clarithromycin, its 14-hydroxy metabolite, and the parent compound erythromycin A.

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MATERIALS AND METHODS

Organisms and growth conditions. *H. pylori* 2765 was inoculated into a tissue culture flask (T-75) containing brain heart infusion broth (Difco) plus 0.1% yeast extract and 10% heat-inactivated horse serum. The flask was placed in an anaerobe jar with Campy Pak, and the jar was incubated at 37°C for 3 days. MICs were determined by agar dilution in Mueller-Hinton agar supplemented with 5% defribrinated horse blood (13). *Escherichia coli* PL2 (*galE relA*) was grown in Lennox L broth base (Gibco) at 37°C.

Preparation of ribosomes. Cells were chilled, collected by centrifugation at 4°C (5,000 × *g* for 5 min), and washed twice in one-half culture volume of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, and phosphate buffer). A final wash with a 1/10 culture volume of 10 mM Tris-HCl containing 4 mM $MgCl_2$, 100 mM KCl, and 10 mM NH_4Cl (pH 7.2) (buffer A) was used before storing the cell pellets at −80°C. Cells were thawed and suspended in buffer A and were lysed by three passages through a French pressure cell at 16,000 lb/in^2. An S30 fraction was collected by centrifugation at 30,000 × *g* for 30 min at 4°C. The supernatant containing the S30 fraction was centrifuged at 100,000 × *g* for 90 min at 4°C to pellet the ribosomes. The ribosomes were suspended in buffer A and were stored at −80°C. Ribosomes from *E. coli* were isolated in a similar manner (9).

Macrolide binding to ribosomes. Macrolide binding to ribosomes at 25°C was performed in buffer A, and ribosomes containing bound macrolide were collected on 0.45-μm-pore-size nitrocellulose filters (Millipore); this was followed by three

washes (3 ml each) with cold 10 mM Tris-HCl (pH 7.2) containing 5 mM $MgCl_2$ and 150 mM KCl (9, 10). Duplicate samples were rapidly collected by filtering (less than 5 s) and then washing as described above; the filters were then transferred to scintillation vials containing InstaGel (United Packard) for radioactivity determination. The entire processing time took less than 30 s. Excess macrolide (to 0.2 μM final concentration) was added to a portion of the remaining reaction mixture during experiments measuring k_1 to ensure that all ribosomes contained bound macrolide (9), thus giving an accurate measure of the total number of ribosomes present. The rate of the forward reaction (formation of the macrolide-ribosome complex) was calculated by using the formula $1/(B_0 - A_0) \ln [A_0(B_0 - x)/B_0(A_0 - x)] = k_1 t$, where B_0 is the concentration of free drug at time zero, A_0 is the concentration of free ribosomes at time zero, x is the concentration of the drug-ribosome complex formed at time t, and k_1 is the forward rate constant (in molar^{-1} minute^{-1}) (9). This equation describes the forward rate for a second-order reaction, but it does not take into account the dissociation rate constant; it will thus be valid only at early times during the reaction. We therefore used initial linear rates to calculate rate constants. The rates of dissociation of the macrolide-ribosome complexes were determined by adding a 100-fold excess of unlabeled macrolide, with subsequent monitoring, in duplicate, of the amount of complex remaining over time (9). The dissociation rate constant was calculated by using the formula $\ln[(RD)/(RD_0)] = -k_{-1}t$, where RD_0 is the concentration of the macrolide-ribosome complex at time zero, RD is the concentration of the macrolide-ribosome complex at time t after the addition of an excess of unlabeled macrolide, and k_{-1} is the reverse rate constant in units of minute^{-1} (9). Data were plotted and rate constants were determined from the slopes for both forward and reverse rate constants. Dissociation constants (K_d) were calculated by the formula $K_d = k_{-1}/k_1$ and are expressed in molar units.

The comparative bindings of unlabeled macrolides were determined by competition with unlabeled macrolides (29). Briefly, ribosomes were incubated with 0.1 μM labeled erythromycin in the presence of various amounts of unlabeled macrolide for 6 h at 25°C. Duplicate samples were processed by filter binding as described above to monitor the amount of labeled macrolide that remained bound. Data were analyzed by use of the following equation: $\log \{[K_0[A]([R_0] - [RA])/[RA] - 1\} = n \log[I] + \log K_I = Y$, where K_0 is the association constant of the radiolabeled macrolide, $[A]$ is the concentration of free labeled macrolide, $[R_0]$ is the total concentration of ribosomes, $[RA]$ is the concentration of the ribosome-labeled macrolide complex, n is the number of inhibitor molecules bound per ribosome, $[I]$ is the concentration of inhibitor, and K_I is the association constant for binding of the inhibitor to the ribosome. Y was plotted as a function of $\log[I]$, and the association constant of the competitor was determined from the x intercept.

Analysis of macrolide binding by sucrose gradient analysis. Forty picomoles of 70S ribosomes were incubated at 37°C for 60 min in 0.5 ml of buffer A containing 400 pmol of [^{14}C]clarithromycin. The chilled sample was layered onto a 15 to 30% (wt/vol) linear sucrose gradient, and the gradient was centrifuged for 5.5 h at 4°C in the SW41 rotor at 38,000 rpm. Gradients were fractionated from the bottom, and fractions (0.3 to 0.4 ml) were collected. Water (0.7 ml) was added to each fraction, and the A_{260} per fraction was recorded. The entire fraction was then added to scintillation vials containing 9 ml of InstaGel (United Packard), and the radioactivity was determined by liquid scintillation counting. A_{260} values were used to quantitate the amounts of ribosomal subunits present and thus estimate the stoichiometry of macrolide binding to ribosomal subunits.

P_u and P_i determinations. Partition coefficients (P_u and P_i) were calculated from known pK_a and K_a values, and octanol/water partitioning data at various pH values by the formula $P_{app} = P_i[H^+/(K_a + H^+)] + P_u[K_a/(K_a + H^+)]$ (28).

Reagents. Erythromycin A, clarithromycin, and 14-hydroxyclarithromycin were prepared at Abbott Laboratories. [*N-methyl*-^{14}C]erythromycin A (50 mCi/mmol), [*N-methyl*-^{14}C] clarithromycin (28 mCi/mmol), and [*N-methyl*-^{14}C]14-hydroxyclarithromycin (15 mCi/mmol) were prepared by the radiosynthesis group in the Biotransformation Section at Abbott Laboratories.

RESULTS AND DISCUSSION

Isolation of *H. pylori* ribosomes. *H. pylori* cells tended to lyse under our standard procedure of washing by centrifugation in buffer A, and thus an alternative washing procedure was used. Cells were washed two times in 1/2 culture volume of PBS prior to a final wash with 1/10 culture volume of buffer A. Approximately 90 A_{260} units were recovered in the supernatant containing the S30 fraction from cells obtained from 100 ml of culture, and 30 to 40 A_{260} units of 70S ribosomes were recovered in the S100 pellet, as assessed by sucrose density gradient centrifugation in the presence of 10 mM Mg^{2+} (data not shown). Ribosomes, 1 A_{260} unit of 70S particles, bound 15 pmol of macrolide at saturation, giving a yield of roughly 500 pmol of ribosomes. Ribosomes were stable at −80°C for at least 2 months, and macrolide binding was not altered after one cycle of freezing-thawing.

Association rate constant of the erythromycin–70S ribosomal subunit complex. The forward rate constant for the association of radiolabeled [*N-methyl*-^{14}C]erythromycin A was determined by measuring the rate of association between macrolide and 70S ribosomes at 25°C (Fig. 1A and B). The rate of the forward reaction (formation of the macrolide-ribosome complex; Fig. 1A) was calculated by plotting $1/(B_0 - A_0) \ln [A_0(B_0 - x)/B_0(A_0 - x)]$ versus time. The slope of the line at the early linear phase is a measure of k_1, the forward rate constant, in molar^{-1} minute^{-1}. The r^2 value over the first 3 min of the reaction (Fig. 1B) was 0.988, giving a slope, and thus a forward rate constant, of 3.3×10^6 M^{-1} min^{-1}. This association rate constant is significantly slower by 1 order of magnitude than those that we previously measured for the interaction of erythromycin with ribosomes from other bacteria (9), demonstrating a significant difference in the structure of the macrolide binding site on *H. pylori* ribosomes.

Dissociation rate constant for the macrolide–70S ribosome complex. Dissociation rate constants were measured by following the exchange of bound ^{14}C-labeled macrolide with a 100-fold excess of unlabeled erythromycin. Dissociation rate constants were calculated from plots of $\ln[(RD)/(RD_0)]$ versus time (Fig. 2). Linear regression analysis gave r^2 values of between 0.973 and 0.999. Dissociation rate constants for erythromycin and clarithromycin were 100 to 200 times slower than the dissociation rate constants that we previously measured for the interaction of erythromycin with ribosomes from the gram-negative bacteria *E. coli* and *Haemophilus influenzae* (9). They were also 30- to 90-fold slower than the dissociation rate constants that we measured for the interaction of erythromycin with ribosomes from the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (9). Figure 2 includes data for the dissociation rate of erythromycin from the *E. coli* ribosome measured simultaneously with *H. pylori* ribosomes, showing a 200-fold faster rate for erythromycin dissociation

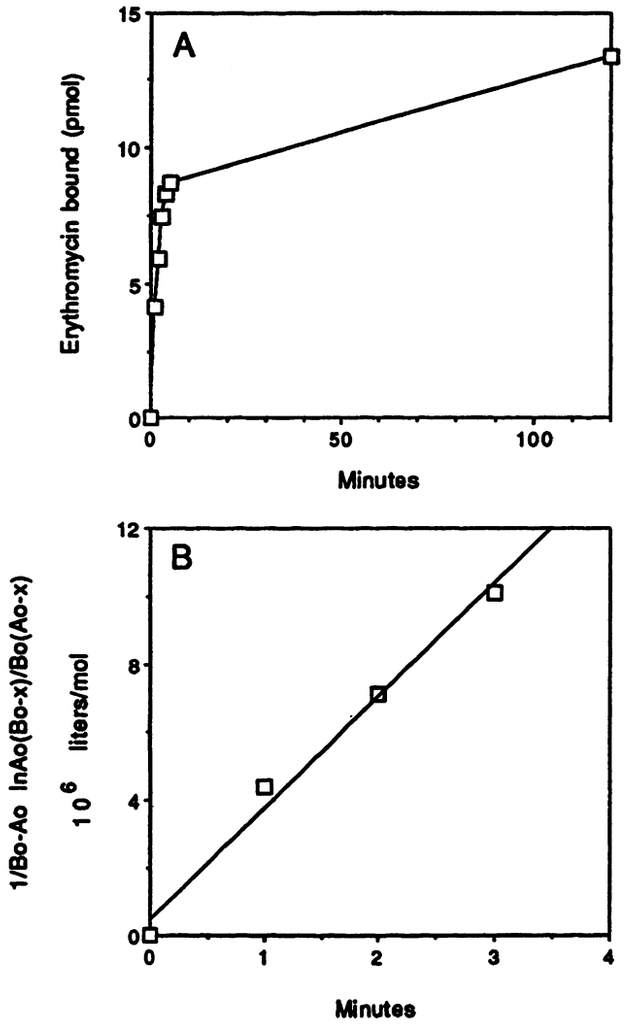

FIG. 1. Rate of formation of the *Helicobacter* ribosome-erythromycin complex. (A) A total of 27 pmol of ribosomes per ml was incubated in buffer A at 25°C, and following the addition of [^{14}C]erythromycin to 0.10 μM (11,000 cpm/ml), samples were removed for filtration binding of the macrolide-ribosome complex. After 5 min, the concentration of [^{14}C]erythromycin was increased to 0.20 μM, and binding was continued until 2 h. (B) Analysis of the early time point in the binding reaction by plotting time versus $1/(B_0 - A_0) \ln [A_0(B_0 - x)/B_0(A_0 - x)]$ (see text). The r^2 value for linear regression was 0.988 by using the data from zero time and the first three datum points. The slope, 3.3×10^6, represents the forward rate constant in molar^{-1} minute^{-1}.

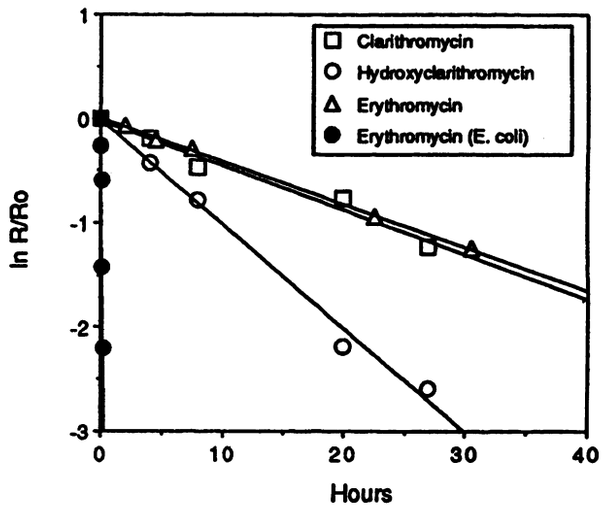

FIG. 2. Analysis of dissociation rates for *H. pylori* and *E. coli* ribosome-macrolide complexes. *Helicobacter* ribosomes (16 to 24 pmol/ml) were incubated with 0.10 μM [^{14}C]clarithromycin, 14-hydroxyclarithromycin, or erythromycin for 3 h at 25°C. A 100-fold excess of unlabeled erythromycin was then added to each reaction mixture, and the samples were removed for filtration binding of the macrolide-ribosome complex. Data were plotted by using $\ln[(RD)/(RD_0)] = -k_{-1}t$, as given in the text. At time zero, the counts per minute bound to *Helicobacter* ribosomes per 1 ml of reaction mixture were 984, 908, and 1,820 cpm for clarithromycin, 14-hydroxyclarithromycin, erythromycin, respectively, and 1,530 cpm/ml for erythromycin bound to *E. coli* ribosomes (14.5 pmol of ribosomes per ml). The value of k_{-1} was determined from the slope.

from *E. coli* ribosomes. The off rate for 14-hydroxyclarithromycin was significantly faster than those for both clarithromycin and erythromycin (half-lives of 7 h versus 16 to 17 h), indicating a significant difference in its mode of binding.

Kinetic constants for macrolide interaction with *H. pylori* ribosomes. The K_d for the interaction of erythromycin with *H. pylori* ribosomes was determined by using the following equation: $K_d = k_{-1}/k_1 = 6.83 \times 10^{-4}/3.3 \times 10^6 = 2.07 \times 10^{-10}$ M (Table 1). The K_d values for clarithromycin, 14-hydroxyclarithromycin, and erythromycin were also determined by competition analysis (29) by using the K_a value of 4.83×10^9 M^{-1} (calculated from $K_a = 1/K_d$). The plot of Y versus log [I] (Fig. 3) yielded r^2 values of 0.998, 0.986, and 0.966, for erythromycin, clarithromycin, and 14-hydroxyclarithromycin, respectively, by linear regression analysis. The K_d value determined by competition (Table 1) for erythromycin was within twofold of the values determined by direct measurement of the forward and reverse rate constants. In addition, the values determined for clarithromycin and 14-hydroxyclarithromycin were 1.15 and 0.91 times, respectively, the value determined for erythromycin. Since the k_{-1} values were determined for all macrolides, the range in kinetic parameters on the basis of two determinations (directly from the forward and reverse rate constants for erythromycin and proportional values determined for clarithromycin and 14-hydroxyclarithromycin and by competition analysis measured for all macrolides) are given in Table 1. The reason for the twofold discrepancy in the value for erythromycin determined by the two methods is unknown.

Although 14-hydroxyclarithromycin had a twofold faster dissociation rate constant compared with that for erythromycin or clarithromycin, the K_d values were nearly identical (Table 1 and Fig. 3). Thus, the association rate constant must be significantly faster for 14-hydroxyclarithromycin (Table 1) in order to achieve the same range of binding affinity. Thus, the 14-hydroxy substitution on the clarithromycin structure altered not only the forward rate constant but also the reverse rate constant, indicating that its interaction with the macrolide binding site was significantly different.

Analysis of [^{14}C]clarithromycin binding to ribosomal subunits. *H. pylori* 70S ribosomes were incubated with [^{14}C]clarithromycin, and ribosomal subunits were separated by sucrose density gradient centrifugation (Fig. 4). Analysis of the A_{260} units under the 50S subunit peak gave a sum of 1.72. Since 1 A_{260} unit of *H. pylori* 70S ribosome bound 15 pmol of macrolide, 1 A_{260} unit of 50S subunit should bind 22 pmol of

TABLE 1. Kinetics of macrolide interaction with ribosomes[a]

Organism and compound	k_{-1}	k_1	K_d
H. pylori			
Erythromycin	6.83×10^{-4}	3.3×10^6 (1.58×10^6)[b]	2.07×10^{-10} (4.31×10^{-10})
Clarithromycin	7.07×10^{-4}	3.05×10^6 (1.46×10^6)	2.32×10^{-10} (4.83×10^{-10})
14-Hydroxyclarithromycin	16.6×10^{-4}	8.47×10^6 (4.06×10^6)	1.96×10^{-10} (4.09×10^{-10})
E. coli[c]			
Erythromycin	0.150	ND[d]	ND
Erythromycin	*0.138*	*6.4×10^7*	*2.20×10^{-9}*

[a] The kinetic constants k_1 (forward rate constant, in molar^{-1} minute^{-1}), k_{-1} (reverse rate constant, in minute^{-1}), and K_d (dissociation constant, in molar) were calculated as described in the text.

[b] Values in parentheses were estimated on the basis of results of competition experiments.

[c] The off rate measured for *E. coli* at the same time as *H. pylori* was 0.150, whereas it was 0.138 when it was determined with a different batch of ribosomes and macrolide. The other values in italics were determined previously (10).

[d] ND, not determined in the current study.

macrolide on the basis of the mass of RNA present in the 50S subunit (i.e., 0.66 the mass of RNA in the 70S ribosome). Thus, 1.72 A_{260} units of 50S subunit should equal 1.72 × 22 pmol/A_{260} = 39 pmol. Analysis of the amount of [^{14}C]clarithromycin associated with the 50S subunit peak gave a value of 42 pmol, thus giving a stoichiometry of 1.11 macrolide molecules bound per 50S subunit. The tight binding of clarithromycin to *H. pylori* ribosomes would allow little dissociation during the 5.5-h centrifugation run at 4°C, and thus stoichiometric binding was expected. There was no discernible binding to the 30S subunit, and unbound macrolide was found at the top of the gradient.

General conclusions. The kinetics of interaction of erythromycin, 14-hydroxyclarithromycin, and clarithromycin with *H. pylori* ribosomes are virtually identical, and thus do not explain the differences in MICs (the MICs of erythromycin, 14-hydroxyclarithromycin, and clarithromycin were 0.06, 0.03, and 0.008 μg/ml, respectively, for *H. pylori* 2765). This order of potency, clarithromycin > 14-hydroxyclarithromycin > erythromycin, with hydroxyclarithromycin being only 1 to 2 dilutions more active than clarithromycin, was also observed with a panel of *H. pylori* strains (8a). Thus, some parameter of the macrolide-bacterium interaction other than the kinetics of the interaction with ribosomes is likely to explain the lower MIC of clarithromycin. Although there was a significant difference between the forward and reverse rates constants for clarithromycin and 14-hydroxyclarithromycin, the K_d values were nearly identical. Again, some parameter other than ribosome binding affinity probably explains the slightly lower MIC of clarithromycin compared with that of 14-hydroxyclarithromycin. The difference between erythromycin and clarithromycin is at the C-6 position on the macrolide ring: —OH for erythromycin

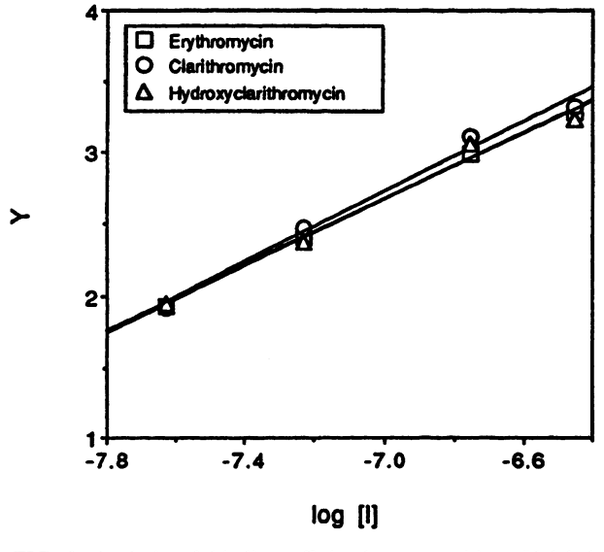

FIG. 3. Analysis of binding affinity by competition. Unlabeled macrolides were used to compete for [^{14}C]erythromycin binding to *H. pylori* ribosomes. Ribosomes (19 pmol/ml) were incubated with [^{14}C]erythromycin (100 nM) and various concentrations of unlabeled competing macrolide for 6 h at 25°C. Samples were processed by filtration binding of the macrolide-ribosome complex. Data were plotted as log [*I*] versus *Y* (see text), and the association constant was determined from the *x* intercept. The control reaction, [^{14}C]erythromycin binding to *H. pylori* ribosomes in the absence of competitor, gave 2,047 cpm ml^{-1} bound to ribosomes.

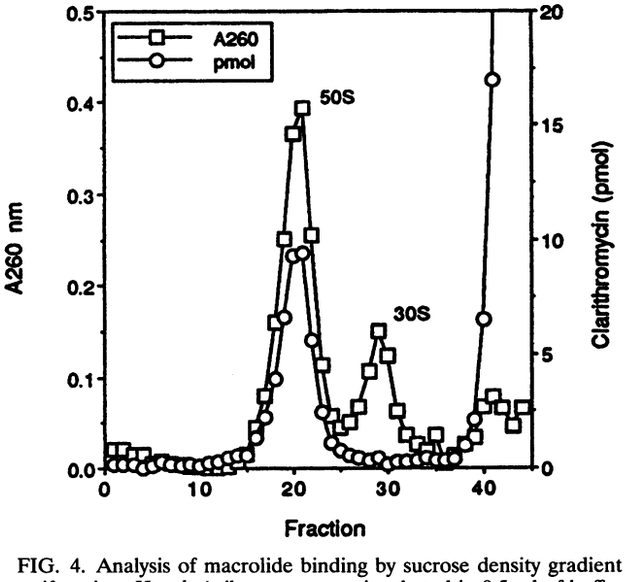

FIG. 4. Analysis of macrolide binding by sucrose density gradient centrifugation. *H. pylori* ribosomes were incubated in 0.5 ml of buffer A containing 400 pmol of [^{14}C]clarithromycin for 60 min at 37°C. The chilled sample was layered onto a 15 to 30% linear sucrose gradient, and the gradient was centrifuged for 5.5 h in the SW41 rotor at 38,000 rpm. The gradient was fractionated from the bottom and was analyzed by measuring the A_{260} and the amount of radioactivity per fraction.

and —OCH_3 for clarithromycin. This change would increase the log P value, and thus the hydrophobicity of clarithromycin. Our experimental values for the log partition coefficient of the unionized (P_u)/ionized (P_i) forms of erythromycin and clarithromycin were 2.91/−0.89 and 3.24/−0.37, respectively. Although we have not experimentally determined values for 14-hydroxyclarithromycin, hydroxylation of clarithromycin at the C-14 position would decrease the log P value by a calculated 0.5 units. Thus, the permeability coefficient of macrolides for the *H. pylori* membrane barrier might be the determining factor in antibacterial potency, with the more hydrophobic clarithromycin having faster entry and thus more potent activity.

Including our current data, reported macrolide affinities range from 1×10^{-6} to 2×10^{-10} M, depending on the macrolide and ribosome used (5, 9, 10, 18, 20, 22, 23). Reported dissociation rate constants (9, 10, 20, 23) for the macrolide-ribosome complex range from 0.150 min^{-1} (half-life, 4.6 min) to 6.83×10^{-4} min^{-1} (half-life, 10^3 min), and reported forward rate constants (9, 10, 20, 23) range from 1×10^5 to 6×10^7 M^{-1} min^{-1}. Thus, there are dramatic differences in the association of macrolides with bacterial ribosomes which likely reflect subtle differences in the precise contacts made between the antibiotic and its binding site (protein and/or RNA contacts). Such differences will likely affect the precise manner of protein synthesis inhibition, including the degree of stimulation of release of peptidyl-tRNA from the A site (19), and thus subsequent physiological perturbation (e.g., the mechanism and degree of cidal activity, the amount of residual protein synthesis, the duration of the postantibiotic effect, and alteration of cellular regulatory processes, etc.). An 11,12 cyclic carbamate derivative of erythromycin with a dissociation rate constant of 0.0005 min^{-1} (10) produced more extensive killing and a longer postantibiotic effect in gram-positive bacteria than erythromycin, for which the dissociation rate constant was 0.067 (8b). In this regard, erythromycin, clarithromycin, and 14-hydroxyclarithromycin showed increased cidal activities against *H. pylori* compared with that of the nonmacrolide protein synthesis inhibitor chloramphenicol (8a), demonstrating that the manner of protein synthesis inhibition can alter the physiological outcome.

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