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Role of Protonated and Neutral Forms of Macrolides in Binding to Ribosomes from Gram-Positive and Gram-Negative Bacteria

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Erythromycin binds to a single site on the bacterial 50S ribosomal subunit and perturbs protein synthesis. However, erythromycin contains desosamine and thus exists in both protonated (>96%) and neutral (<4%) forms at physiological pH because of the pK_a of the dimethylamino group. We therefore examined the relative roles of both forms in binding to ribosomes isolated from two species each of gram-positive and gram-negative bacteria. We developed a system to directly measure the forward (association) rate constant of formation of the macrolide-ribosome complex, and we have measured both the forward and reverse (dissociation) rate constants as a function of pH. Forward rate constants and binding affinity did not correlate with pH when the interaction of erythromycin with ribosomes from both gram-positive and gram-negative bacteria was examined, demonstrating that the protonated form of this macrolide binds to ribosomes. Conversely, the neutral form of macrolide cannot be the sole binding species and appears to bind with the same kinetics as the protonated form. Forward rate constants were 3- to 4-fold greater at physiological pH, and binding affinity calculated from rate constants was 5- to 10-fold greater than previously estimated. Similar results were obtained with azithromycin, a novel 15-membered macrolide that contains an additional tertiary amine in the macrolide ring. Ribosome- and macrolide-specific kinetic parameters were demonstrated at neutral pH and may be related to the potency of the two macrolides against gram-positive and gram-negative bacteria.

Erythromycin has been studied and used for antibacterial chemotherapy for over 30 years. Initial efforts were directed towards determination of its mode of action and efficacy in clearing bacterial infections. These early investigations showed that erythromycin was a very safe and effective agent for treating infection due to susceptible bacteria and that its mode of action involved binding to bacterial 50S ribosomal subunits (11, 13) with resultant perturbation of protein synthesis (1, 12). Although the details regarding perturbation of ribosome function are still not entirely understood, binding to a single site on the 50S ribosomal subunit has been firmly established by independent analysis (7–10, 13, 20).

Once the existence of the binding site on the 50S subunit was demonstrated, studies shifted to a more detailed evaluation of the kinetic parameters involved. Initial determinations of dissociation constants for the erythromycin-ribosome complex ranged from 10^{-6} to 10^{-8} M (3, 4, 10, 13, 16, 20), and the specific dissociation (reverse) rate constant of the [^{14}C]erythromycin-*Escherichia coli* ribosome complex was 0.15 min^{-1} (16). The study that found the latter result gave only a calculated association (forward) rate constant, 1.5×10^7 liters mol^{-1} min^{-1}, because the forward rate was too fast to be measured under the experimental conditions used. The measured forward rate constants were 1.25×10^5 and 1.36×10^6 liters mol^{-1} min^{-1} for 50S subunits and 70S ribosomes, respectively, while the reverse rate constants were 0.138 and 0.589 min^{-1} for 50S subunits and 70S ribosomes, respectively, when the interaction of a fluorescent (5-fluorescein isothiocyanate) derivative of (9S)-erythromycylamine with ribosomes in real time was studied (7, 8). Although its reaction kinetics differed significantly from those of the parent molecule, erythromycin A, the fluorescent derivative did bind to a single site with second-order reaction kinetics and dissociated with first-order reaction kinetics. The interaction of erythromycin with *E. coli* ribosomes was also studied indirectly by its ability to compete with the naturally fluorescent antibiotic virginiamycin type B. The calculated forward and reverse rate constants were 1.92×10^7 liters mol^{-1} min^{-1} and 0.264 min^{-1}, respectively (3).

Erythromycin contains a dimethylamino group on the desosamine sugar and has a pK_a of 8.6 to 8.9 in H_2O (18, 21); thus, at physiological pH it exists in both protonated (>96 to 98%) and neutral (2 to <4%) forms. Since reactant concentrations are key terms in the analysis of reaction kinetic constants, it is difficult to evaluate data from the literature without first knowing the relative roles of the neutral and protonated forms of macrolide in binding to bacterial ribosomes. Determining whether the protonated or neutral form of erythromycin, or both, binds to the 50S ribosomal subunit is important not only for the analysis of interaction kinetics, but also for understanding the mode of action of erythromycin. If both forms bind, one must consider the possibility that each causes a different perturbation of ribosome function, a possibility that has apparently been considered only once in the literature (12). That study revealed a strong correlation between pH and potency of inhibition of in vitro protein synthesis for the basic macrolides erythromycin and oleandomycin, ranging from a stimulation of protein synthesis below pH 7 to progressively increasing inhibition of protein synthesis up to pH 8.5. These results led Mao and Weigand to conclude that the neutral macrolide form was the inhibitory species.

In contrast to the lack of information about the roles of protonated and neutral forms of erythromycin in interaction with bacterial ribosomes, the roles of both forms in transport across the bacterial membrane have been investigated. Increasing extracellular pH increases the antimicrobial potency of basic (18) but not neutral (14) macrolides and concurrently increases the rate of uptake into the bacterial cell (2). These data are most easily explained if the neutral

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form is considered to cross the cell membrane while the protonated form is severely restricted. The physiochemical factor giving rise to this selectivity at the cell membrane barrier is most likely the sphere of tightly bound water which is known to surround positively charged amines and restrict their ability to partition into nonpolar solvents, i.e., the phospholipid bilayer structure of cell membranes. The partition coefficient and rate of transfer of erythromycin and other macrolides between aqueous and modal organic solvents are dependent on the pH of the aqueous phase (21; S. Borodkin, unpublished data), and these data support the conclusion that only the neutral macrolide species effectively partitions into the organic phase. If the hypotheses described above are correct, erythromycin could concentrate within a cellular compartment which was maintained at a pH less than the pH bathing the compartment because of ion trapping; i.e., the influx rate would be greater than the efflux rate because of differing concentrations of the neutral, membrane-permeating macrolide species. Recent evidence (6) supports this hypothesis, at least for eucaryotic cells.

We have developed a method to directly monitor the forward rate constant for the formation of the macrolide-ribosome complex and have measured both the forward and reverse rate constants as well as the extent of binding for the interaction of two macrolides, erythromycin and azithromycin, with ribosomes prepared from gram-positive and gram-negative bacteria. In this report, we show that (i) the protonated forms of these macrolides bind to ribosomes; (ii) consequently, the neutral form cannot be the sole species which binds to ribosomes, and the neutral form apparently binds with kinetics similar to those of the protonated form; (iii) both protonated and neutral forms appear to dissociate from ribosomes with similar reverse rate constants; and (iv) the reaction kinetics of the two macrolides at physiological pH vary depending on which bacterial ribosomes are examined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bacillus subtilis* DB104, *Staphylococcus aureus* 3000, *Haemophilus influenzae* 19418, and *E. coli* PL2 (*galE relA*) from our laboratory collection were used in the preparation of ribosomes. Bacteria were grown at 37°C in Lennox L broth base (GIBCO Diagnostics) (except for *H. influenzae*, which was grown in brain heart infusion plus 5% Fildes enrichment [Difco Laboratories]) to late log phase and harvested by centrifugation.

Purification of bacterial ribosomes. Ribosomes were prepared from *B. subtilis*, *S. aureus*, and *E. coli* by differential centrifugation as described elsewhere (4). Since our attempts to isolate ribosomes from *H. influenzae* by differential centrifugation resulted in poor yields of intact ribosomes (less than 10%), an alternative method employing Sephacryl S-200 chromatography was used (5). In all cases, ribosomes were stored at −80°C in 10 mM Tris hydrochloride buffer (pH 7.5) containing 10 mM $MgCl_2$, 60 mM NH_4Cl, 5 mM EDTA, 5 mM mercaptoethanol, and 10% glycerol (vol/vol).

Analysis of macrolide binding to ribosomes. Macrolide binding to ribosomes at 25°C took place in 10 mM Tris hydrochloride (pH as given) containing 4 mM $MgCl_2$, 100 mM KCl, and 10 mM NH_4Cl, and ribosomes containing bound macrolide were collected on 0.45-μm-pore-size nitrocellulose filters (Millipore Corp.) and washed three times (3 ml each) with cold 10 mM Tris hydrochloride (pH as given) containing 5 mM $MgCl_2$ and 150 mM KCl, as previously described (16). This is the basic method used previously to analyze erythromycin binding to *E. coli* ribosomes at pH 7.2 (16). The rate of the forward reaction (formation of the macrolide-ribosome complex) was calculated as described previously (4). Ribosome and macrolide concentrations were reduced to 40 to 60 nM in order to slow the rate of complex formation. Triplicate 5-ml samples were rapidly collected by filtering the mixtures through 0.45-μm-pore-size nitrocellulose filters for less than 5 s and then washing as described above. The entire process took less than 30 s. Excess macrolide (1 μmol) was then added to a portion of the remaining reaction mixture to ensure that all ribosomes contained bound macrolide, thus giving an accurate measure of the total number of ribosomes present. Rates of dissociation of 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. The dissociation rate constant was calculated as described elsewhere (4). Data were plotted and rate constants were determined from slopes for both forward and reverse rate constants. Since the forward rate declines with time because of dissociation of complex which occurs during the course of the binding reaction, initial rates were used in the calculation of k_1 (the forward rate constant). Dissociation constants (K_d) were calculated by the formula $K_d = k_{-1}/k_1$, where k_{-1} is the reverse rate constant, and are expressed in terms of molarity. K_d determinations were also attempted by the method of Scatchard (19). The pH of the ribosome-binding buffer was adjusted with HCl or NaOH, and control measurements demonstrated that the addition of macrolide and ribosomes did not change the pH. The experimental error in sampling macrolide bound to ribosomes was 5.5% ± 3.3% and ranged from 2.2 to 8.7%. The R values for the straight lines defining the association and dissociation rate constants were between 0.93 and 1.0.

Determination of macrolide pK_a. pK_as were determined by aqueous titration with approximately 1 mg of macrolide per ml. The pK_a of erythromycin was also measured by nuclear magnetic resonance (NMR) in the ribosome-binding buffer as follows. Erythromycin A (1.33 mg) was dissolved in 0.5 ml of an H_2O-^2H_2O (9:1) solution containing Tris hydrochloride buffer (10 mM; pH 7.2), NH_4Cl (5 mM), KCl (50 mM), and $MgCl_2$ (4 mM). The pH of the sample was adjusted with a concentrated solution of NaOH and measured on a digital pH meter (Corning Glass Works) equipped with a microprobe. NMR spectra were recorded at 30°C on an AM 500 NMR spectrometer (Bruker). A sweep width of 5,000 Hz and a 90° proton pulse of 7.5 μs were employed. The water resonance was irradiated during the delay (2.5 s) between scans in order to suppress the large solvent signal. The proton chemical shifts are reported relative to those of 3-(trimethylsilyl)propionic acid.

Sources of macrolides and radiochemical methods. [*N-methyl*-^{3}H]erythromycin A (40 mCi/mmol) and [*N-methyl*-^{14}C]azithromycin (6.59 mCi/mmol) were synthesized at Abbott Laboratories. Radiochemical purity monitored during the course of these studies was >98%. The correction for filter quenching of [^{3}H]erythromycin was determined by flame combustion analysis to ^3H_2O with a Packard combustion analyzer (United Packard). Unlabeled erythromycin A was prepared as a fermentation product, while unlabeled azithromycin was prepared by chemical synthesis. Chalcomycin was obtained from Sigma Chemical Co.

RESULTS AND DISCUSSION

Analysis of kinetic constants. Although the kinetics of macrolide binding to ribosomes have been reported in the

literature, we felt it important to examine the relative roles of protonated and neutral forms of basic macrolides in binding to ribosomes. The predominant form at pH 7.2 (>96 to 98%) is protonated, while a minor fraction (2 to <4%) is neutral for a basic macrolide such as erythromycin (pK_a = 8.6 to 8.9, measured by aqueous titration). Since the concentration of the actual binding reactant is a critical factor in calculating binding kinetics, we felt it necessary to determine the relative roles of the protonated and neutral macrolide species in binding to bacterial ribosomes.

We felt that the most direct approach would be to measure forward and reverse rates for the interaction of macrolide with ribosomes as a function of pH and to calculate the respective rate constants. As a prelude to these studies, we first determined the pK_a of erythromycin in the actual buffer to be used for binding experiments. The pK_a determined by NMR was 9.1, which is close to that determined in a pure aqueous system (8.6 to 8.9). Thus, we could proceed to monitor the forward rate constant at various pHs under conditions in which we were confident about our calculations of the fractions of macrolide present in the protonated and neutral forms.

We measured the forward rate for the macrolide-ribosome interaction simply by diluting the reactants to the nanomolar range (Fig. 1A), thus allowing calculation of forward rate constants (Fig. 1B; see above). The rationale of the experiments discussed below is simple. As we increase the pH at which binding is monitored, the fraction of neutral macrolide increases dramatically, with a concurrent decrease in the fraction present in the protonated form. If only one of the forms binds, the forward rate constant should vary predictably with pH. If a protonation or deprotonation step is involved in binding of the neutral or protonated species, respectively, binding affinity should also vary predictably with pH.

Analysis of kinetic constants at neutral pH. Forward rate constants for the two macrolides examined ranged from 2.5 $\times 10^7$ to 6.4 $\times 10^7$ liters mol^{-1} min^{-1} at pH 7.2 (Table 1); these constants are significantly less than those indicative of association reactions controlled mainly by diffusion (9). These data imply that a conformational change occurs in the ribosome during macrolide binding. We observed an even smaller forward rate constant (4.5 $\times 10^5$ liters mol^{-1} min^{-1}) for the binding of a novel macrolide derivative which is also capable of binding to macrolide-lincosamide-streptogramin B-resistant ribosomes (4). In this case, our data indicated that significant conformational change in the ribosome occurred upon binding of this derivative, decreasing both the forward and reverse rate constants compared with those characteristic of the parent compound, erythromycin.

The dissociation rates for the macrolide-ribosome complexes were measured after the addition of excess unlabeled macrolide; in all cases, dissociation followed a first-order rate at pH 7.2, and the rate constants were calculated. The dissociation rate constants calculated ranged from 0.138 to 0.063 min^{-1} for erythromycin and from 0.05 to 0.019 min^{-1} for azithromycin, with erythromycin dissociating faster from gram-negative ribosomes than from gram-positive ribosomes. In addition, azithromycin dissociated more slowly from gram-negative ribosomes than erythromycin did.

The K_d values calculated for the binding affinities of the two macrolides at pH 7.2 with the four ribosome preparations are also given in Table 1. K_d values for erythromycin binding to gram-negative ribosomes were 5.8- and 7.9-fold greater (*H. influenzae* and *E. coli* ribosomes, respectively) than for azithromycin binding. A binding affinity of azithromycin greater than that of erythromycin was suggested previously on the basis of competition binding to *E. coli* ribosomes (16), but rate constants were not reported. Our data show that forward rate constants are similar for the interactions of these two macrolides with ribosomes isolated from both *H. influenzae* and *E. coli* and that the increased binding affinity of azithromycin is due to a smaller dissociation rate constant. The slower dissociation rate for azithromycin is probably due to a conformation at the ribosome-binding site slightly different from that of erythromycin rather than to any direct role of the additional charged amine in the macrolide ring. In contrast, both macrolides bind with similar affinity to gram-positive ribosomes. The K_d values we obtained indicated tighter binding to ribosomes than previously reported (3, 10, 13, 16).

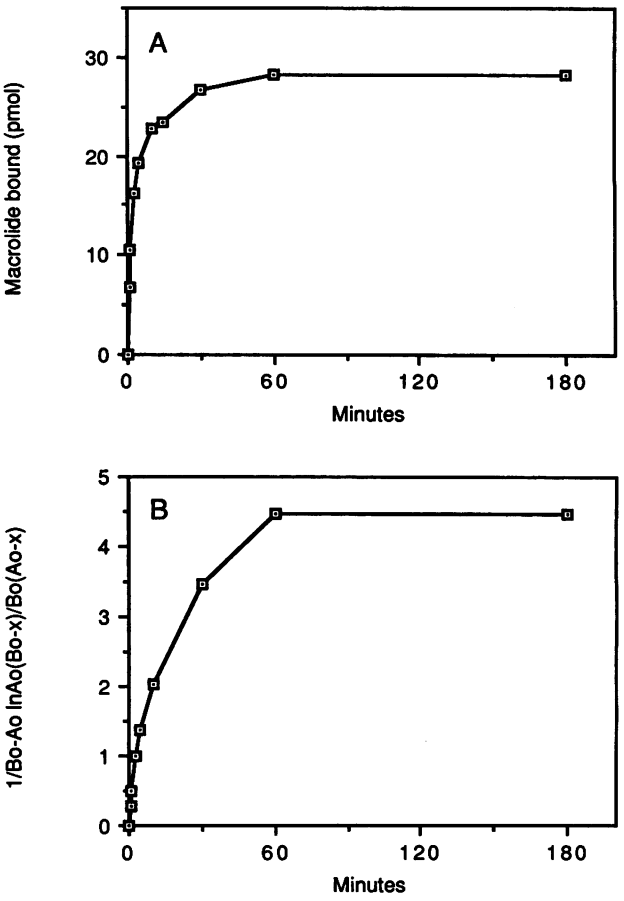

FIG. 1. Kinetic analysis of formation of macrolide-ribosome complex. Ribosomes isolated from *H. influenzae* were incubated with radiolabeled azithromycin, and the ribosome-macrolide complex was isolated by filter binding. (A) Rate of complex formation was monitored with time. (B) Data from panel A were analyzed as described in the text for calculation of the forward rate constant. All forward rate constants were calculated from data collected in the first 1 to 2 min of reaction, during which the slope is linear. Ao, Concentration of free ribosomes at time zero; Bo, concentration of free drug at time zero; x, concentration of drug-ribosome complex at time *t*.

Analysis of forward rate constants at different pHs. Forward rate constants for erythromycin binding to ribosomes isolated from two gram-positive and two gram-negative bacterial species varied only slightly over a pH range of 6 to

TABLE 1. Kinetics of macrolide interaction with ribosomes at pH 7.2

Ribosome source	Kinetic constants for[a]:					
	Erythromycin			Azithromycin		
	k_1	k_{-1}	K_d	k_1	k_{-1}	K_d
H. influenzae	6.0×10^7	0.106	1.8×10^{-9}	5.2×10^7	0.016	3.1×10^{-10}
E. coli	6.4×10^7	0.138	2.2×10^{-9}	6.4×10^7	0.018	2.8×10^{-10}
B. subtilis	4.9×10^7	0.063	1.3×10^{-9}	3.1×10^7	0.050	1.6×10^{-9}
S. aureus	2.5×10^7	0.024	9.6×10^{-10}	2.8×10^7	0.019	6.8×10^{-10}

[a] All reactions were performed in ribosome-binding buffer at pH 7.2, and samples were taken in triplicate for determination of the amount of macrolide bound to ribosomes by filter binding. Kinetic constants: k_1, forward rate constant (liters per mole per minute); k_{-1}, reverse rate constant (per minute); K_d, dissociation constant (molar concentration).

9 (Table 2). The rate constant was always slightly larger at pH 7.2 (Table 1) but never varied by a factor of more than 2.8. In contrast, the amount of neutral macrolide species present over the ranges of pH studied (Table 2) varied by a factor of 200 to 400, while the amount of protonated macrolide varied by a factor of greater than 2. The protonated form of macrolide must bind to ribosomes, since the slight changes in forward rate constants did not correlate with the fraction of macrolide present in the neutral form. Conversely, the neutral form cannot be the sole species which binds to ribosomes and apparently binds with similar kinetics. We were unable to conduct detailed studies of the interaction of the neutral macrolide species in the absence of a signal from the protonated form because ribosomes were unstable at the high pH (pH 10) required to reduce the amount of the protonated form to 10% of the total. However, there are several biologically active macrolides (lankamycin, kujimycin, chalcomycin, neutramycin, 23672-RP, and aldgamycin) which lack a dimethylamino group on the sugar attached to C-5 in the macrolide ring (14, 15). Chalcomycin, which contains a neutral methoxy group in place of a dimethylamino group, bound to gram-positive bacterial ribosomes with approximately one-fifth the affinity of the protonated form of erythromycin, showing that a protonated group at this position is not absolutely required (data not shown). Many ionizable groups on the ribosome should also be affected by changes in pH, and this may explain the slight changes observed in forward rate constants with various pHs.

TABLE 2. Kinetics of macrolide interaction at low and high pHs

Ribosome source and pH level[a]	Erythromycin concn (nM)[b]		Kinetic constants[c]		
	Protonated	Neutral	k_1	k_{-1}	K_d
H. influenzae					
Low	8.57	0.026	2.4×10^7	0.080	3.33×10^{-9}
High	3.76	4.84	3.0×10^7	0.105	3.50×10^{-9}
E. coli					
Low	10.29	0.013	5.4×10^7	0.164	3.04×10^{-9}
High	4.61	5.69	4.0×10^7	0.166	4.15×10^{-9}
B. subtilis					
Low	10.29	0.013	1.7×10^7	0.058	3.41×10^{-9}
High	4.61	5.69	2.0×10^7	0.117	5.85×10^{-9}
S. aureus					
Low	10.29	0.013	1.2×10^7	0.029	2.42×10^{-9}
High	4.61	5.69	1.4×10^7	0.051	3.64×10^{-9}

[a] pHs of the reaction mixtures were 6.53 and 8.99 (*H. influenzae*) and 6.23 and 9.19 (*E. coli*, *B. subtilis*, and *S. aureus*).

[b] Amounts of erythromycin in the neutral and protonated forms were calculated from the pK_as determined in ribosome-binding buffer by using the Henderson-Hasselbach equation.

[c] k_1, Forward rate constant (liters per mole per minute); k_{-1}, reverse rate constant (per minute); K_d, dissociation constant (molar concentration).

Similar results were obtained during analysis of the binding of azithromycin to ribosomes prepared from *H. influenzae*. The pK_as of the two ionizable nitrogen groups in azithromycin were very close during aqueous titration (8.8 to 8.9); thus, a value of 8.85 was used to calculate the fractions of neutral and protonated forms present. Limited quantities of this macrolide precluded pK_a determinations by NMR in the ribosome binding buffer; however, since the pK_as of erythromycin were very close in the two systems, any similar difference for azithromycin would not introduce significant error in data interpretation. The forward rate constants were 5.8×10^7, 5.2×10^7, and 5.0×10^7 liters mol^{-1} min^{-1} at pHs 6.5, 7.2, and 9.0, respectively, while the concentrations of protonated and neutral forms varied by factors of 2.4 and 150, respectively, over this pH range. We conclude that the forward rate constants for binding of the neutral and protonated forms of azithromycin are similar, if not identical, for *H. influenzae* ribosomes.

Analysis of dissociation rate constants at various pHs. Since both the neutral and protonated forms of the two macrolides tested seem able to bind to ribosomes, we believed that both forms would be bound to the ribosome in a ratio reflective of the solution pH. Dissociation rate constants for the macrolide-ribosome complex were previously determined at neutral pH; these data thus applied only to the protonated form, which represented >98% of the total macrolide present. We proceeded to examine the dissociation rate constants for erythromycin and azithromycin as functions of pH and species of bacterial ribosome.

The effect of pH on the kinetics of dissociation of the two macrolides from ribosomes was macrolide dependent and ribosome dependent. The dissociation rate constants for erythromycin from gram-negative ribosomes were relatively independent of pH, while pH dependence was observed for dissociation from gram-positive ribosomes (Table 2). The dissociation rate for erythromycin release from gram-positive ribosomes increased approximately twofold when the pH was increased from 7.2 to 9.2. Since >98% of erythromycin is protonated at pH 7.2 but only 45% is protonated at pH 9.2, we considered the possibility that the dissociation rate kinetics reflect combined dissociation rates for the two forms of macrolide bound (neutral and protonated), which would occur only if the bound conformations of the two forms differed enough to effect a difference in dissociation rate. Assuming that the dissociation rate constant for the protonated form (0.058 min^{-1}) was independent of pH, the

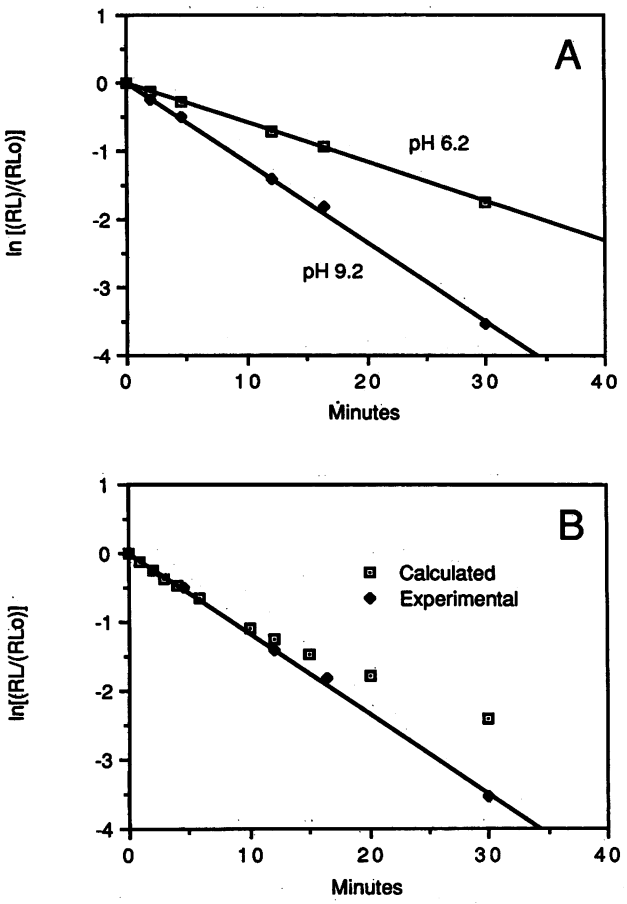

FIG. 2. Kinetic analysis of dissociation of erythromycin-ribosome complex. Ribosomes isolated from *B. subtilis* were incubated with radiolabeled macrolide for 2 h (equilibrium binding), and a 100-fold excess of unlabeled macrolide was then added. The amount of macrolide remaining bound to ribosomes with time was monitored by filter binding. Data were analyzed as described in the text for calculation of the dissociation rate constant. (A) Dissociation of erythromycin at pHs 6.2 and 9.2. (B) Comparison of experimental data at pH 9.2 with calculated data for a two-component system in which the protonated (45% of the total) and neutral (55% of the total) forms dissociated with rate constants of 0.058 and 0.20 min^{-1}, respectively. See text for details. RL, Concentration of drug-ribosome complex at time *t*; RLo, concentration of drug-ribosome complex at time zero.

calculated rate for dissociation of the neutral form would have to be 0.20 min^{-1} in order to satisfy the new half time observed for dissociation of the complex at pH 9.2, at which the protonated and neutral forms represent 45 and 55% of the total, respectively. We calculated the dissociation curve expected for such a two-component system with these values (Fig. 2B) and found that the curve was clearly biphasic. The data for erythromycin dissociation from *B. subtilis* ribosomes gave a simple first-order plot at both pH 6.2 and pH 9.2 (Fig. 2A) consistent with equivalent dissociation rates for both the neutral and protonated macrolide forms. Similar data were gathered for azithromycin interaction with ribosomes from *B. subtilis* (data not shown).

General conclusions. The association and dissociation of macrolides can be described by the general formula D + R ↔ DR, where D represents a macrolide and R represents a ribosome; the forward and reverse rate constants are k_1 and k_2, respectively. However, since macrolides which contain

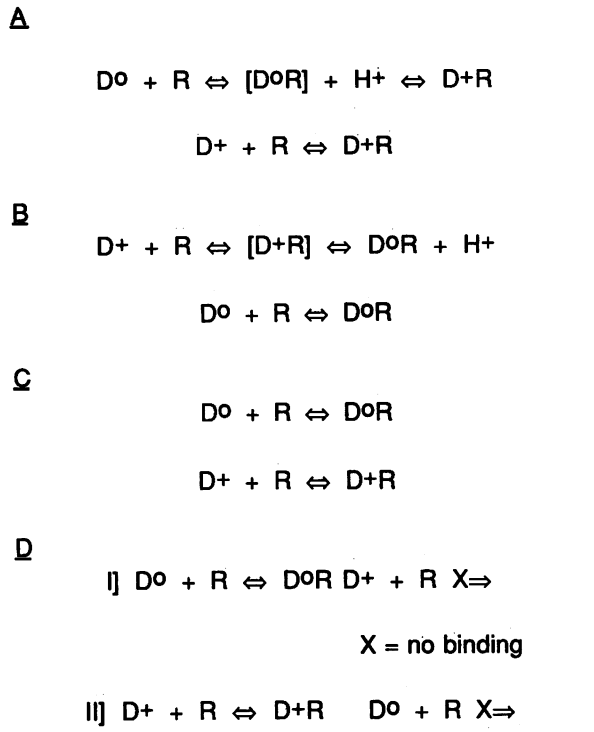

FIG. 3. Possible models for the roles of neutral and protonated forms of macrolides in binding to bacterial ribosomes. D^0, Neutral macrolide; D^+, protonated macrolide; R, ribosome; X⇒, no binding. Complexes in brackets represent loosely bound intermediates.

desosamine exist in both protonated and neutral forms, the involvement of both forms in the binding reaction must be considered. We considered several possibilities. (i) Both forms bind similarly to ribosomes, and thus both $D^0 + R \leftrightarrow D^0R$ and $D^+ + R \leftrightarrow D^+R$ reactions occur (where D^0 and D^+ are the neutral and protonated forms of the macrolide, respectively), with k_1 and k_2 being similar for both D^0 and D^+ (Fig. 3C). (ii) Both protonated and neutral forms bind to ribosomes with similar affinity and thus with only slightly different rate constants (similar to Fig. 3C). (iii) Both protonated and neutral forms initiate binding to ribosomes, but only the protonated (Fig. 3A) or neutral (Fig. 3B) form is present in the tightly bound macrolide-ribosome complex. This would involve an intermediate in the binding of either form of the macrolide (Fig. 3). In case A (Fig. 3A), only the protonated form is considered to bind directly into the tight complex, with the neutral form evolving from a loosely bound intermediate which locks into the tightly bound form after protonation. In case B (Fig. 3B), only the neutral form is considered to bind directly into the tight complex, with the protonated form evolving from a loosely bound intermediate which locks into the tightly bound form after deprotonation. (iv) Only the neutral or only the protonated macrolide form initiates binding and performs the final tight binding (Fig. 3D).

Since the forward rate constant does not vary significantly or predictably with pH, both neutral and protonated forms appear able to initiate binding to form the tight complex which is measured by filter binding. This rules out case D. In addition, inasmuch as the binding affinity (K_d; Tables 1 and 2) also did not vary significantly or predictably with pH, we can rule out cases A and B (Fig. 3) on the basis of thermodynamic principles. The binding affinity should have

varied with pH because a proton is involved in the transition of the intermediate to the tightly bound form for both cases A and B. Thus, we conclude that both the protonated and the neutral forms of the two macrolides tested bind to the ribosome with similar affinities (Fig. 3C). Although we did observe a pH-dependent increase in the dissociation rates of macrolides from gram-positive ribosomes, the reaction still appeared to be first order, and the data clearly did not fit a hypothetical two-component system in which the bound protonated and neutral macrolide species dissociated with significantly different kinetics. The increase in dissociation rate was likely due to a pH-dependent alteration in the gram-positive ribosome which affected dissociation of both the protonated and neutral forms.

Compared with erythromycin, azithromycin shows increased inhibitory potency against gram-negative bacteria but not gram-positive bacteria (17). We have recently ruled out any effect of the membrane proton motive force on the uptake of both erythromycin and azithromycin into *H. influenzae* (J. O. Capobianco and R. C. Goldman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, A-138, p. 24), demonstrating that the additional positive charge on azithromycin does not stimulate uptake by a proton motive force-regulated pathway. Such an effect would be difficult to support, even on thermodynamic grounds, in the absence of a membrane carrier because of the strongly bound hydration shell present around positively charged amines. The inward driving force due to proton motive force (intracellular negative) would be largely offset by the requirement for dehydration before the phospholipid bilayer of the plasma membrane is entered. Since azithromycin has an increased potency for gram-negative but not gram-positive bacteria, our data implicate ribosome-binding affinity as a factor related to the MIC. However, preliminary experiments indicate that ribosome-binding affinity is not the sole factor involved in the increased potency of azithromycin against gram-negative bacteria, other important factors being the ability to cross the outer membrane permeability barrier and access to intracellular ribosomes (R. Goldman, C. Doran, and J. Capobianco, manuscript in preparation).

The results of our studies pose many additional questions. What is the actual pK_a of the dimethylamino group, and what is the distribution of protonated and neutral macrolide forms in the cytoplasm of intact bacteria? The neutral intracellular pH of most pathogens suggests that erythromycin and azithromycin exist primarily in the protonated form once they gain access to the cytoplasm and thus that most ribosome-binding sites are occupied by the protonated macrolide form. Once the ribosome engages in protein synthesis, what is the relative affinity (dissociation rate constant) of the two species of bound macrolide? Do the neutral and protonated forms affect ribosome function similarly? A previous report (12) demonstrated that the ability of erythromycin to inhibit in vitro protein synthesis by ribosomes isolated from *S. aureus* increases with increasing pH. Mao and Weigand concluded that only the neutral form of macrolide efficiently inhibited protein synthesis. We observed greater inhibition at pH 8.5 than at pH 7.5 when erythromycin was used to inhibit poly(A)-directed polylysine synthesis in S30 extracts prepared from *B. subtilis* (unpublished data). These results pose an interesting question inasmuch as our data indicate that both the neutral and protonated forms of basic macrolides bind efficiently to ribosomes. We are currently extending these studies to answer the questions posed above.

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