Chemical Characterization of Ethambutol Binding to *Mycobacterium smegmatis*

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Recent studies showed that critical binding of ethambutol to *Mycobacterium smegmatis* was both inhibited and reversed by ions. This apparent ion-susceptible characteristic suggested that ethambutol is held at critical sites by electrostatic bonds. Dissociation constant, pH, and ionic strength studies were designed to further characterize ethambutol binding. *M. smegmatis* was grown in Sauton synthetic liquid medium (pH 7.4) under aerated conditions at 37 C. The pH or ionic strength of the medium was modified to meet the needs of particular experiments. Titration data revealed that ethambutol dihydrochloride has two apparent dissociation constants ($pK_{a1} = 6.35$, $pK_{a2} = 9.35$). Uptake experiments, in which pH was varied, showed that dihydrochloride and free base ethambutol were bound to a greater extent than the monohydrochloride. However, dihydrochloride and free base binding were not related to biological activity. Ethambutol exerted its maximal growth inhibitory effect at pH values near neutrality, where it exists primarily as the monohydrochloride and showed minimal binding. The increased ethambutol binding observed at pH 7.4 in media of lowered ionic strength was consistent with growth studies showing a reduction in the minimal inhibitory growth concentration in such media. However, nonspecific as well as critical binding was enhanced at low ionic strength. We conclude that binding of ethambutol by *M. smegmatis* involves a heterogeneous group of drug binding sites, only one of which is directly related to biological activity. Although nothing is known about the ethambutol target site itself, critical binding of the drug seems to require the single positively charged monohydrochloride form. Both hydrogen bonds and ionic linkages are probably involved.

Ethambutol uptake by *Mycobacterium smegmatis* and the relationship of uptake to growth inhibition have been under investigation in this laboratory (2, 3). Kinetic experiments revealed a diphasic uptake pattern (2), in contrast to the apparent one-step reaction reported earlier by Forbes et al. (7). However, phase one uptake in our system had characteristics of the rapid binding described by the other workers, and growth inhibition depended on this primary interaction rather than on secondary uptake. Experiments in which ^{14}C -labeled ethambutol was used in conjunction with specific washing techniques showed that drug bound as a result of the primary reaction was comprised of at least two fractions, based on ion sensitivity (2). It was originally thought that the ion-stable fraction represented critical binding. However, further studies (3) indicated that critical binding was inhibited at high ionic strength and was

sensitive to salt solution washing. It appears to reside, therefore, in the ion-susceptible fraction.

In an interaction between ethambutol and *M. smegmatis*, one would anticipate that charge distribution on the two imino groups in the drug molecule (Fig. 1) plays a central role in primary binding. Because ethambutol is highly stereospecific (9, 10), the hydroxyl groups probably also play important roles. In this paper dissociation constant, pH, and ionic strength experiments are presented that suggest that binding of ethambutol is, by and large, electrostatic in nature and involves several different kinds of binding sites, only one of which is directly related to biological activity.

MATERIALS AND METHODS

Organism. *M. smegmatis* strain H-607 was provided by the United States-Japan Cooperative Medical Science Program, National Institute of Allergy

and Infectious Diseases. The organism was grown at 37 C with aeration (rotary shaking at 150 rpm) in 100- or 200-ml volumes of modified Sauton synthetic liquid medium containing 0.02% Tween 80 (4).

Experimental media. For the pH experiments unadjusted Sauton medium was titrated to the desired pH with 5.0 N NaOH. Steam sterilization did not significantly alter the pH values. In the ionic strength studies standard Sauton medium, pH 7.4 (without Tween 80), was either diluted with deionized water or fortified with NaCl to either decrease or increase the strength, respectively. Tween 80 was then added to give a final concentration of 0.02%, and the pH was readjusted to 7.4. The specific conductance of each medium was measured to determine actual ionic strength in terms of NaCl equivalents.

Ethambutol. Ethambutol [*d*-2,2'-(ethylenedimino)-di-1-butanol dihydrochloride] was a gift from Lederle Laboratories, Pearl River, N.Y. ^{14}C -labeled ethambutol dihydrochloride (specific activity, 6.92 μCi per mg of free base drug) was prepared by the method of Wilkinson et al. (9). Purity of the synthesized compound was ascertained by thin-layer chromatography. Biological activity was confirmed with *M. smegmatis*. Both stock and working solutions of the agents were prepared in deionized water and, where appropriate, sterilized by filtration. All concentrations are expressed in terms of free base drug.

Instrumentation. Cell concentrations in Sauton medium were estimated turbidimetrically with a Coleman Junior spectrophotometer (Coleman Instrument Co., Maywood, Ill.). Readings were made at 600 nm in tubes (18 by 150 mm). An optical density (OD) of 0.2 was equivalent to 0.28 mg (dry weight) of cells per ml.

Cell-bound ^{14}C activity was assayed with a Beckman LS-100 liquid scintillation counter (Beckman Instruments, Palo Alto, Calif.). Samples were placed in vials containing 10 ml of liquid scintillation fluid prepared by adding 42 ml of Liquifluor (Pilot Chemicals, Inc., Watertown, Mass.) and 40 g of Cab-o-sil (Packard Instrument Co., Downers Grove, Ill.) to 1 liter of toluene. The vials were shaken vigorously and counted to an error of 3%. All data were corrected for background (about 50 counts/min) and normalized to 25 mg (dry weight) of cells.

Dissociation constants for ethambutol dihydrochloride were determined by titration of the drug with standard NaOH in a Radiometer Titrator type TTT 1c equipped with Autoburette type ABULa (The London Co., Westlake, Ohio).

To estimate the actual ionic strengths of Sauton medium and its various modifications, specific conductances were determined on the media and related to a standard conductivity curve based on NaCl solutions of known ionic strength (μ). The salt concentration required to prepare each standard solution was calculated from the equation: $\mu = \Sigma mZ^2/2$, where m = ion molarity and Z = ion charge. Specific conductance (SC) was calculated from the equation: $\text{SC} = K/Rm$, where K = cell constant (cm^{-1}) and Rm = measured cell resistance in ohms. Measured cell resistance was determined with an LKB 5300 A

Conductolyzer (LKB-Produkter AB, Stockholm, Sweden) at 25 C.

^{14}C ethambutol uptake experiments. Overnight log-phase cultures were combined and adjusted to 0.28 mg (dry weight) of cells per ml with standard Sauton medium. The cells contained in separate 75-ml portions of adjusted culture were collected on bacteriological filter membranes (diameter, 47 mm; pore size, 0.8 μm) and washed with deionized water. Each cell mass was then scraped from the filter and dispersed in 75 ml of either standard Sauton medium or medium appropriately modified from the standpoint of pH or ionic strength. After a 1-h incubation with labeled drug at 37 C and rotary shaking, the cells were harvested again on filter membranes, washed with deionized water, and prepared for counting as previously described (2).

Growth assays. For the pH studies, overnight log-phase cultures were diluted to an OD of 0.2 with Sauton medium. Portions of the adjusted suspension were filtered and washed with deionized water. The harvested cells were scraped off the filter membranes and dispersed in Sauton media of varying pH to give an OD of 0.05. Volumes (75 ml) of diluted suspension were placed in 500-ml nephelo-culture flasks equipped with side arms (19 by 130 mm; Bellco Glass, Inc., Vineland, N.J.). The cultures were incubated with drug at 37 C and rotary shaking. Growth was monitored turbidimetrically. In the ionic strength experiments, drug was added to 50-ml portions of modified media in 125-ml Erlenmeyer flasks. Each flask was inoculated with 0.75 ml of a heavy stock culture (OD approximately 0.5) and incubated at 37 C with aeration. At 18 h, growth was measured turbidimetrically.

RESULTS

Titration data presented in Fig. 1 show that ethambutol has two apparent dissociation constants, $\text{pK}_{a1} = 6.35$ for the dihydrochloride and $\text{pK}_{a2} = 9.35$ for the monohydrochloride. These results are in good agreement with values of 6.1 and 9.2 determined by the staff at Lederle Laboratories (personal communication). Therefore, in standard Sauton medium (pH 7.4) the monohydrochloride form of the drug is predominant.

The influence of charge distribution, or pH, on total binding of ^{14}C ethambutol by *M. smegmatis* is shown in Fig. 2. Surprisingly, the amount of label bound at either pH extreme (6.0 and 9.5) was about twice that occurring in the more physiological range. However, this troughing phenomenon is not unprecedented. Chang and Flaks, for example, observed a similar effect in the course of studies on dihydrostreptomycin binding by *Escherichia coli* ribosomes (5). The binding data in Fig. 2 are even more interesting when considered together with pH growth studies presented in Fig. 3 and

FIG. 1. Determination of apparent dissociation constants for ethambutol. Ethambutol dihydrochloride (0.100 mmol in 4.0 ml of deionized water) was titrated with 0.0500 N NaOH. The curve is based on two separate determinations.

4. Control cells grew equally well over the pH range examined. Ethambutol exhibited its greatest growth inhibitory effects at pH values approaching neutrality, where drug binding was minimal.

From the standpoint of ionic strength, the strong electrolytes in Sauton medium are of major importance. A calculated value of μ for the medium based on MgSO_4 , K_2HPO_4 , and NaOH is 0.057. Although the medium is a relatively simple synthetic mixture, it seemed probable that the various components influenced the ionization and physiological activity of one another. To account for suppression of ionization, specific conductances were determined for Sauton medium and its various modifications. The values obtained were related to the standard sodium chloride conductivity curve shown in Fig. 5. Based on this curve, the ionic strength of Sauton medium was 0.030 (± 0.002) rather than 0.057.

We showed earlier that total binding of [^{14}C]ethambutol by *M. smegmatis* cells in ordinary Sauton medium was markedly inhibited when the medium was fortified with certain

FIG. 2. Enhanced binding of [^{14}C]ethambutol by *M. smegmatis* cells incubated in Sauton medium adjusted to either an acid or an alkaline pH. [^{14}C]ethambutol concentration, 0.2 $\mu\text{g/ml}$. Incubation time, 1 h.

salts (3). Binding studies at ionic strengths less than that of ordinary Sauton medium (diluted medium) are shown in Fig. 6. As expected, an inverse relationship between ionic strength and

FIG. 3. Reduced biological activity of ethambutol in Sauton medium adjusted to acid or alkaline pH values that permitted growth of *M. smegmatis* in the absence of the drug. The control cultures contained no drug.

drug binding was observed. In medium diluted 1:8 with deionized water ($\mu = 0.004$), binding by the cells was approximately six times greater than in regular Sauton medium ($\mu = 0.030$). Consistent with the binding data, growth experiments showed that the minimal inhibitory concentration (MIC) of ethambutol was shifted downward as ionic strength was decreased (Fig. 7). At μ values ≤ 0.016 , all three concentrations of ethambutol (0.05, 0.1, and 0.2 $\mu\text{g/ml}$) were equally effective as growth inhibitors. As μ was increased, however, biological activity of the drug was diminished until none of the concentrations provided significant bacteriostasis.

DISCUSSION

Critical binding of ethambutol by *M. smegmatis* (i.e., binding that plays a direct role in growth inhibition) appears to involve physicochemical adsorption of the drug at sites near

the cell surface (2). In view of the strict stereospecificity displayed by ethambutol (9, 10) and the overall composition of the drug molecule, it is likely that a particular combination of the imino and hydroxyl groups is responsible for binding. Consistent with this notion, the marked sensitivity of the interaction to inhibition and reversal by ions (2, 3) suggested that ethambutol is held at critical cell sites by electrostatic attractions. Earlier workers reached a similar conclusion from streptomycin binding studies with *E. coli* (1, 6). Titration data revealed that near neutral pH ethambutol exists predominantly as the monohydrochloride, bearing a single positive charge on one of the two imino groups. Thus, under normal growth conditions in Sauton medium (pH 7.4) the charged imino group on the drug may function in ionic bonding with the cell, and the free base imino group as well as one or both

FIG. 4. Relationship between Sauton medium pH and the biological effectiveness of ethambutol on *M. smegmatis*. The control cultures contained no drug. Growth was assayed turbidimetrically after 8.5 h of incubation. Time zero OD was approximately 0.05.

FIG. 5. Standard curve relating specific conductance to ionic strength. Specific conductances were determined on a series of aqueous NaCl solutions of known ionic strength.

hydroxyl groups could be involved in hydrogen bonding.

M. smegmatis grew as well at pH 6.0 and 8.5 as it did at 7.4, but ethambutol was not nearly as effective at the two pH extremes as it was near neutrality. In spite of markedly reduced growth inhibitory activity at pH 6.0, where the drug exists primarily as the doubly charged cation, bound ethambutol was more than twice that measured at pH 7.4. These data suggest the existence of binding sites unrelated to biological activity, which possibly involve only ionic linkages. At pH 8.5, where we would

expect a small fraction of drug to exist as uncharged or nonionized free base, the growth inhibitory action of ethambutol again was reduced, but total binding was nearly identical to that measured at 7.4. Above pH 8.5 growth studies were not possible. However, at values approaching 9.4, where approximately a 50:50

FIG. 6. Enhanced binding of [^{14}C]ethambutol by *M. smegmatis* cells in media of decreasing ionic strength. Each dilution of Sauton medium supported similar growth for at least 16 h (see Fig. 7). [^{14}C]ethambutol concentration, 0.2 µg/ml. Incubation, time, 1 h.

FIG. 7. Enhanced biological activity of ethambutol on *M. smegmatis* in response to decreasing ionic strength. Standard Sauton medium was either diluted with water or supplemented with NaCl to achieve the various ionic strengths. For regular Sauton medium, $\mu = 0.028$. Growth was assayed turbidimetrically after 16 h of incubation. Time zero OD was 0.005 to 0.01.

mixture of the monohydrochloride and free base forms would exist, binding of the drug was about twice that observed at pH 7.4. Therefore, uncharged free base drug appears to bind at another type of site unrelated to biological activity. Hydrogen bonding alone may be involved in this case.

As reported earlier (3), total binding of [^{14}C]ethambutol was inhibited approximately 50% when the ionic strength of Sauton medium was increased with NaCl. In view of these findings and related studies by Forbes et al. (8), it was not surprising that reduction of ionic strength by dilution of Sauton medium gave rise to marked increases in drug binding. Since the MIC of ethambutol was reduced in low ionic strength media at pH 7.4, critical binding appeared to be favored under such conditions. The predominant form of ethambutol at pH 7.4 is the monohydrochloride, and it is therefore reasonable to suppose that the increased binding observed involved drug in this singly charged state. Other data, however, indicated that nonspecific as well as critical binding was enhanced in low ionic strength media. For example, at 0.2 μg of [^{14}C]ethambutol per ml, binding by cells in Sauton medium was only one-sixth of that observed in medium diluted 1:8. Yet strong indistinguishable growth inhibitions were observed in both media. Therefore, with drug concentrations above the ordinary MIC, much of the binding occurring at low ionic strength appears unrelated to biological activity. This binding is indicative of still another type of ethambutol binding site.

The marked decrease in binding observed when pH was increased from 6.0 to 7.4 does not appear to be simply an ionic strength effect. The difference in measured ionic strengths between Sauton medium adjusted to 6.0 and 7.4 with NaOH was very small ($\mu = 0.023$ versus 0.030). An examination of Fig. 6 shows that, at a constant pH of 7.4, binding was nearly the same at these two slightly different ionic strengths.

From results obtained in this investigation, we conclude that binding of ethambutol by *M. smegmatis* involves several different types of drug binding sites, only one of which is directly related to biological activity. These studies emphasize that uptake of a chemotherapeutic

agent by susceptible organisms is not necessarily proportional to effectiveness. At present, information regarding the subcellular fraction or structural component responsible for critical ethambutol binding is lacking and, of course, nothing is known about the specific chemical groups and stereochemical configurations involved on the target site itself. However, based on structure-activity studies (9, 10) and on our results suggesting that critical binding requires the singly charged monohydrochloride form of ethambutol, we can speculate that both hydrogen bonding and ionic linkages are involved. The charge distribution on the imino groups appears to be of primary importance, since absence of the hydroxyl groups resulted in reduced but not complete loss of activity (9).

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