Slow Binding Inhibition of Phospho-N-acetyl muramyl-pentapeptide-translocase (Escherichia coli) by Mureidomycin A*

(Received for publication, July 5, 1995, and in revised form, January 17, 1996)

Philip E. Brandish†, Martin K. Burnham§, John T. Lonsdale¶, Robert Southgate‡, Masatoshi Inukai, and Timothy D. H. Bugg**

From the †Department of Chemistry, Southampton University, Highfield, Southampton SO17 1BJ, United Kingdom, §SmithKline Beecham Pharmaceuticals, Great Burgh, Epsom KT18 5QX, United Kingdom, ¶SmithKline Beecham Pharmaceuticals, Brookham Park, Betchworth, Surrey RH3 7AA, United Kingdom, and §Biomedical Research Laboratories, Sankyo Co., Ltd., 1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140, Japan

Enzymes of the membrane cycle of reactions in bacterial peptidoglycan biosynthesis remain as unexploited potential targets for antibiotic agents. The first of these enzymes, phospho-N-acetyl muramyl-pentapeptide-translocase (EC 2.7.8.13), has been overexpressed in Escherichia coli and solubilized from particulate fractions. The work of W. A. Weppner and F. C. Neuhaus ([1977] J. Biol. Chem. 252, 2296–2303) has been extended to establish a usable routine fluorescence-based continuous assay for solubilized preparations. This assay has been used in the characterization of the natural product, mureidomycin A as a potent slow binding inhibitor of the enzyme with K_i and K_i* of 36 nm and 2 nm, respectively.

Enzymes responsible for the biosynthesis of the peptidoglycan component of the bacterial cell wall are well preceded targets for antibiotics. (1) The worldwide emergence of bacterial strains resistant to current antibiotics necessitates the development of new antimicrobial agents (2, 3). Phospho-N-acetyl muramyl-pentapeptide-translocase (also translocase I) catalyzes the first step in the membrane cycle of peptidoglycan biosynthesis, namely the transfer of phospho-N-acetyl muramyl-L-Ala-g-D-Glu-m-DAP-o-Ala-o-Ala from uridine 5'-monophosphate (UMP) to a membrane-bound lipid carrier, undecaprenyl phosphate (see Fig. 1) (4, 5). This enzyme is encoded by the mraY gene in Escherichia coli. This gene has been cloned and sequenced; examination of the inferred amino acid sequence indicates that the encoded enzyme is an integral membrane protein whose molecular mass is 39.5 kDa (6). The lipid-linked product of MraY is further elaborated by attachment of an N-acetylglucosamine unit and the precursor is somehow flipped across the membrane and incorporated into peptidoglycan. This and other such lipid-linked cycles have been reviewed (7). No commercial antibiotics in current use are directed against translocase I. This enzyme represents a target for novel antibacterial agents which is as yet unexploited.

Since recently, the only known inhibitors of this step of peptidoglycan biosynthesis were ticunacycin, which is known also to inhibit mammalian glycoprotein biosynthesis and other lipid-linked glycosyl transfer reactions (8), and amphotericin, which chelates undecaprenyl-P in the presence of Ca2+ (9, 10). In recent years two new classes of natural products have been characterized as potent and specific inhibitors of this step in peptidoglycan biosynthesis, the mureidomycins and the liposidomycins (11–14). Both classes of compound share a uridine nucleoside moiety found in the substrate UDP-MurNAC-pentapeptide, but beyond this there is little obvious similarity to the substrates of translocase I (see Fig. 2). The presence of the common uridine moiety suggests a similar mode of action for this class of molecules.

The mureidomycins are a class of novel peptidyl nucleoside antibiotics isolated from Streptomyces flavidovirens SANK 60486 which show selective antipseudomonal activity (minimal inhibitory concentration values of 0.1–3.1 μg/ml), while not being toxic in mice (15). Mureidomycin A has been demonstrated to inhibit translocase I activity in particular preparations from Pseudomonas aeruginosa using a radiochemical assay (IC50 0.05 μg/ml), but not to significantly inhibit formation of lipid-linked N-acetylmuramylglycosamine for teichoic acid synthesis in Bacillus subtilis (IC50 > 100 μg/ml) or dolichol-linked precursors for glycoprotein biosynthesis in a mammalian system (11). The antibacterial potency of mureidomycin A and its novel structure prompted us to begin an investigation into the molecular mechanism of action of this antibiotic. Here we report the characterization of mureidomycin A as a slow-binding inhibitor of solubilized translocase I from Escherichia coli using a convenient fluorescence enhancement continuous assay.

EXPERIMENTAL PROCEDURES

Materials—Mureidomycin A was isolated as described previously (16). Dodecaprenyl phosphate and phosphatidylglycerol were obtained from Sigma. Other chemicals and media were commercially available. Protein was determined using the Smith BCA assay (17).

Construction of Expression Vector pBROC525—An 8.6-kb KpnI fragment carrying the mraY gene was subcloned using a DNA preparation made from Kohara phage 110 into the KpnI site of pUC19 to furnish pBROC508 (18). MluI digestion of pBROC508 provided a much smaller 1.3-kb fragment of DNA encoding mraY. MluI overhangs were filled in by treatment with T4 polymerase and the blunt-ended fragment re-cloned into the SmaI site of pUC19 to give pBROC511, where the direction of transcription of the mraY gene was found to be opposite to that of lacZ. Subcloning the 1.3-kb AflIII/BamHI fragment of pBROC511 into the NcoI/BamHI sites of pTrc99A (Pharmacia Biotech Inc.) gave us pBROC525 where expression of the mraY gene from the trc promoter is regulated by lacIq. The AflIII restriction site in the mraY gene in pBROC511 is situated directly at the translation start site.

Preparation of Phospho-N-acetyl muramyl-pentapeptide-translocase—E. coli J M 109 (pBROC525) was grown in LB media at 37°C to OD600 nm 1.7, IPTG added to 0.2 mM, and cells harvested after 1.5 h

*This work was supported by a CASE award (to P.E.B.) from EPSRC and SmithKline Beecham Pharmaceuticals. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
**To whom correspondence should be addressed. Tel.: 01703-593816; Fax: 01703-593781.

The abbreviations used are: DAP, diaminopimelic acid; dansyl, 5-dimethylaminoanaphthalene-1-sulfonic; IPTG, isopropyl-1-thio-β-D-galactopyranoside; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; dPP, dansyl-UDP-MurNAc-pentapeptide; kb, kilobase pair(s).

Preparation of Phospho-N-acetyl muramyl-pentapeptide-translocase—E. coli J M 109 (pBROC525) was grown in LB media at 37°C to OD600 nm 1.7, IPTG added to 0.2 mM, and cells harvested after 1.5 h
further growth. Cells were opened using an air-driven cell disrupter, whole cells and debris were collected by centrifugation, and membrane fragments were collected by ultracentrifugation (105,000 g, 30 min). The pellet was resuspended in 50 mM Tris, pH 7.5, 2 mM β-mercaptoethanol, 1 mM MgCl₂, 1 M KCl and stirred for 1 h at 4°C to strip away peripherally associated proteins. Inclusion of MgCl₂ in preparative buffers increased the yield of activity 1.5–2-fold. Membrane fragments were collected again, resuspended in buffer without KCl, flash frozen in liquid nitrogen, and stored at −20°C. Enzyme was solubilized from membranes at a protein concentration of 4 mg/ml in 50 mM Tris, pH 7.5, 2 mM β-mercaptoethanol, 1 mM MgCl₂, 1% v/v Triton X-100, 20% glycerol with stirring for 1 h at 4°C. Insoluble material was removed by ultracentrifugation (105,000 g, 30 min).

Preparation of Fluorescent Substrate—UDP-MurNAc-pentapeptide was isolated from cells of B. subtilis W23 based on methodology devised by Lugtenberg et al. (19) and Flouret et al. (20). UDP-MurNAc-pentapeptide was accumulated in B. subtilis by treatment with chloramphenicol and vancomycin in cell wall synthesis medium II described previously (19) and extracted from cells with cold 5% w/v trichloroacetic acid. This crude preparation was chromatographed on Bio-Gel P2, and fractions containing only the desired uridine nucleotide were identified using reverse-phase high performance liquid chromatography as described previously (20). UDP-MurNAc-α-Ala-γ-D-Glu-m-DAP-(N-dansyl)-α-Ala-α-Ala was prepared by treatment of the natural substrate with dansyl chloride in 50% aqueous acetone essentially as described Weppner and Neuhaus (21). The compound purified by gel filtration on Bio-Gel P2 was characterized by UV spectroscopy and time-of-flight mass spectrometry; it was stored as a stock solution at −70°C.

Radiochemical Assay for Translocase I—This assay is a modification of the method of Tanaka et al. (9). UDP-MurNAc-α-Ala-γ-D-Glu-m-DAP-[14C]-o-Ala-[14C]-o-Ala was a generous gift from SmithKline Beecham Pharmaceuticals. Reaction mixtures contained 100 mM Tris, pH 7.5, 25 mM MgCl₂, 10 nCi of [14C]UDP-MurNAc-pentapeptide and unlabeled UDP-MurNAc-pentapeptide (final concentration, 14 µM) and 20 µg of particulate protein or 20–50 µg of solubilized protein in a 50-µl volume. In assays with particulate enzyme, heptaprenyl phosphate was added as a dispersion in a 5-µl aliquot of 0.1% Triton X-100. Where solubilized preparations were used, detergent was included in the assay mixture such that the final concentration remained constant throughout the experiments (0.1% Triton X-100). Lipids in organic solvent were placed in a microcentrifuge tube, the solvent was removed with a stream of nitrogen, and the residue was dissolved in the aliquot of solubilized enzyme to be assayed. Assays were stopped by the addition of 50 µl of 6 M pyridinium acetate, pH 4.2. Product was extracted with 100 µl of 1-butanol and quantified in Optiphase “HiSafe” 3.

Fluorescence Enhancement Assay for Translocase I—Fluorescence measurements were made using a Perkin-Elmer LS-3 fluorescence spectrophotometer equipped with an 0.5-cm light path silica quartz cuvette (Perkin-Elmer Corp.) maintained at 30°C. Aliquots of dodecaprenyl phosphate and phosphatidylglycerol (Sigma) were placed in a microcentrifuge tube, and the solvent was removed with a stream of nitrogen. Lipids were taken up in detergent solubilized enzyme as in the radiochemical method and equilibrated to 30°C. Assay constituents were added in the following order: 150 µl of assay buffer containing 200 mM Tris, pH 7.5, 100 mM KCl, 50 mM MgCl₂; 80 µl of H₂O; 60 µl of solubilized translocase (20–200 µg of protein) + lipids (final concentrations, 40 µM and 100 µM for dodecaprenyl phosphate and phosphatidylglycerol, respectively). Lipid-linked intermediate I formation was started by addition of 10 µl of stock fluorescent substrate, giving a final

![UDP-MurNAc-pentapeptide](image1)

**Fig. 1. Transfer of phospho-N-acetylmuramyl-pentapeptide to undecaprenyl phosphate catalyzed by translocase I.**

![UDP-MurNAc-pentapeptide](image2)

**Fig. 2. Chemical structures of translocase I substrates and inhibitors.**
concentration of 105 μM. In inhibition experiments, inhibitor was added simultaneously with the fluorescent substrate. To monitor the reverse reaction, the forward reaction was allowed to go to equilibrium before addition of 0.4 mM UMP, giving rise to a decrease in fluorescence emission. Increase in fluorescence emission concomitant with formation of lipid-linked product was monitored at 535 nm (excitation at 340 nm).

RESULTS

Overexpression of E. coli Translocase I—Preliminary investigation of the levels of translocase I activity in wild-type E. coli J M105 revealed a low specific activity of 20 pmol/min/mg protein in the absence of exogenous lipid acceptor using a radiochemical assay. Ikeda et al. (6) have shown previously that expression of the mraY gene in multicopy plasmids in E. coli leads to stimulation of translocase I activity in particulate fractions. Accordingly, an overexpression vector pBROC525 was constructed in order to increase the specific activities of enzyme preparations. This plasmid is derived from pTrc99A (Pharmacia) and contains a 1.3-kb DNA fragment harboring the E. coli mraY gene under the control of the trc promoter. The specific activity of particulate enzyme from E. coli J M109 (pBROC525) was 276 pmol/min/mg of protein in the absence of exogenous lipid acceptor. Specific activity was increased 2-fold by induction with IPTG in the late logarithmic phase (OD500 = 1.7) over early (OD500 = 0.6) induction, with 90 min of growth after induction in both cases. Using the fluorescence enhancement assay (see below), J M109 (pBROC525) gave a specific activity of 11.9 units/min/mg compared to 0.42 units/min/mg for J M109 (pTrc99A), corresponding to a 28-fold overproduction of enzyme activity in solubilized preparations under optimized conditions. Examination by SDS-PAGE of particulate membrane fractions of E. coli J M109 (pTrc99A) and E. coli J M109 (pBROC525) induced with IPTG revealed no new bands corresponding to the mraY gene product in the range 35–45 kDa.

Solubilization of Translocase I—Previous work on translocase I activity in Staphylococcus aureus (Copenhagen) showed that activity could be extracted from membrane preparations with lauryl sarcosinate (22). A range of detergents including lauryl sarcosinate, sodium deoxycholate, CHAPS, EDTA were found to give good yields of activity (70–123%) after centrifugation at 100,000 × g. Of these five, the most stable extract with the highest yield of activity was obtained with Triton X-100 at a detergent/protein ratio of 2.5 ml/mg protein. These conditions were used for further experiments.

Under optimized conditions, preparations of J M109 (pBROC525) solubilized with 1% Triton X-100 at 4 mg of protein/ml gave specific activities of 1–2 nmol/min/mg. Attempts to purify the solubilized enzyme by a wide range of conventional or affinity chromatographic methods gave >95% loss of enzyme activity. No loss of activity was observed upon gel filtration on Sephadex G-75, suggesting that a “factor” is being lost during

FIG. 3. Lineweaver-Burk determinations of Km values. Panel A, Km determination for dPP. Panel B, Km determination for dodecaprenyl phosphate (DP) (see footnote 2). Initial rate measurements were made using the fluorescence enhancement assay as described in the experimental section; first varying DP concentration with dPP present at 105 μM, then varying dPP concentration with DP present at 40 μM. Phosphatidylglycerol was constant at 100 μg/ml. Km values were determined from the slope using the reciprocal forms of the initial forward rate equation for a Ping Pong Bi Bi system (32).

FIG. 4. Effect of Triton X-100 (panel A) and MgCl2 (panel B) in the fluorescence enhancement assay. Assays were carried out as under “Experimental Procedures” except that the concentration of MgCl2 or Triton X-100 was varied accordingly.
can accept heptaprenyl phosphate and dodecaprenyl phosphate followed Michaelis-Menten kinetics indicating that the enzyme enhancement assay, measurement of rate increase in rate in both assay systems. Using the fluorescence is due to product inhibition and approach to equilibrium. Diluted enzyme remained linear over an increase of ~2.5 fluorescence units.

adsorptive chromatography. Experiments are in progress to identify this factor.

Fluorescence Enhancement Assay—Weppner and Neuhaus have shown that UDP-MurNac-L-Ala-γ-d-Glu-L-Lys-(N*-dansyl)-d-Ala-d-Ala is a substrate for nascent peptidoglycan synthesis in membrane preparations from S. aureus Copenhagen (21). The S. aureus translocase processes the dansylated material with slightly higher catalytic efficiency to the natural substrate (21). It was also demonstrated that this substrate could be used for a continuous fluorescence-based assay for translocase I activity. Such an assay would make possible kinetic studies which until now have not been accessible using existing stopped radiochemical assays (11, 21, 23). The methodology developed by Neuhaus and co-workers has been extended to allow fast, convenient continuous assay of detergent solubilized translocase I from E. coli.

In this assay system dansylated substrate is converted into lipid-linked product which resides within detergent micelles rather than the bulk aqueous compartment, resulting in a measurable change in fluorescence emission. Conversion of dansylated substrate to lipid-linked product resulted in a blue shift of the fluorescence emission maximum (exciting at 340 nm) from 565 nm to 535 nm, and a fluorescence enhancement at equilibrium of approximately two-fold. In our hands this assay method is of similar sensitivity to the previous radiochemical assay since the amount of enzyme required to generate 20 pmol of 14C-lipid-linked butanol-extractable product in a 3-min assay gave a change in fluorescence of 3.24 units in a 3-min assay.

Fluorescence enhancement was found to be directly proportional to protein concentration, and linear at low conversions for up to 30 min (data not shown). At higher enzyme concentrations nonlinearity was observed upon approach to chemical equilibrium. Activity is expressed as increase in units of fluorescence emission per minute at 30 °C. Since the quantum yield of the fluorophore under the conditions employed is not known, this unit is arbitrary.

Characterization of Solubilized Translocase I—Previous studies with S. aureus translocase I have relied on endogenous undecaprenyl phosphate co-solubilized with the enzyme (22) or on addition of a crude lipid preparation to enzyme depleted of lipids by extraction with organic solvents (24). We have found that addition of commercially available heptaprenyl (C35) phosphate or dodecaprenyl (C40) phosphate gave a >10-fold increase in rate in both assay systems. Using the fluorescence enhancement assay, measurement of rate versus concentration followed Michaelis-Menten kinetics indicating that the enzyme can accept heptaprenyl phosphate and dodecaprenyl phosphate as substrates. Michaelis-Menten kinetics were also observed with the fluorescent substrate. The rate in the absence of added prenyl phosphate was about one fourth of that with 40 mM dodecaprenyl phosphate under optimized conditions (data not shown). Thus the rate due to endogenous undecaprenyl phosphate is small.

Using the fluorescence enhancement assay the K_m for danyl-UDP-MurNac-pentapeptide (dPP) has been measured at 19 ± 3 μM and the apparent K_m for dodecaprenyl-P was 13 ± 3 μM with a V_max of 4.7 units/min (see Fig. 3). For heptaprenyl-P the apparent K_m was measured at 19 ± 5 μM (data not shown) with a corresponding V_max of 1.7 units/min. The relative K_m and V_max values indicate that dodecaprenyl-P is a more efficient substrate for translocase I than heptaprenyl-P, indicating that the enzyme is selective for the larger substrate which is closer in chain length to the natural substrate, undecaprenyl-P. Consequently, dodecaprenyl-P was used in subsequent kinetic studies.

Dependence of activity in the fluorescence enhancement assay upon magnesium ions and detergent concentration was examined (see Fig. 4). The results reveal a requirement for >10 mM Mg^{2+}, with maximal activity at 40 mM Mg^{2+}. Activity was stimulated 2-fold by the inclusion of 50 mM KCl in the assay. This is consistent with the results obtained with the S. aureus enzyme (25). Assays with other divalent metal ions revealed that Mg^{2+} could be replaced only by Mn^{2+}. The optimal concentration for Mn^{2+} was 1 mM, but activity was 2.5-fold less than with the optimal concentration of Mg^{2+}. No activity was observed using Ni^{2+}, Ca^{2+} and Zn^{2+}. Optimal detergent concentration in the assay was found at 0.25% Triton X-100.

Activity was stimulated 5-10-fold by inclusion of 100 μg/ml phosphatidyl-glycerol in both radiochemical and fluorescence enhancement assays, but not by phosphatidylethanolamine at the same concentration. Specific activation by phosphatidylylycerol is preceded by the case of UDP-GlcNAcDolichyl phosphate GlcNAc-1-phosphate transferase from pig aorta, a eukaryotic enzyme which catalyzes a similar phosphosugar transfer reaction (26). Activation by phosphatidylylycerol and other phospholipids has also previously been observed with a gel-filtered preparation of the S. aureus translocase I activity in 1% Triton X-100, using a radiochemical exchange assay (27). It is not possible to say whether this activation is due to maintenance of the structural integrity of the protein in detergent solution or perhaps more efficient solubilization of the lipid substrate.

Inhibition of Translocase I by Mureidomycin A—Consistent with the work of Isono et al. (28) with ether-treated cells of E.
coli NIHJ, mureidomycin A inhibited solubilized E. coli translocas I in the radiochemical assay with IC50 < 100 nM. Using the fluorescence enhancement assay, mureidomycin A was found to inhibit translocas I activity in a time-dependent fashion. A family of progress curves with increasing inhibitor concentration is illustrated in Fig. 5. The inhibition is characterized by an initial decreased rate, followed by a transition to a final steady-state rate over 2-3 min. When mureidomycin A was included in assays of the reverse reaction as described under “Experimental Procedures” the same biphasic time-course was observed (data not shown).

The observed time-dependent inhibition is consistent either with irreversible or slow binding inhibition. In order to distinguish between irreversible and slow binding inhibition, assays containing 100 nM mureidomycin A were allowed to proceed for varying lengths of time, and intrinsic enzyme activity measured by addition of 0.4 mM UMP and subsequent decrease in fluorescence intensity due to the reverse reaction. The observed rate of reverse reaction upon addition of UMP was independent of incubation time over 5-50 min, indicating that no irreversible enzyme inactivation is taking place. No loss of potency of the inhibitor was observed upon prolonged storage in aqueous medium, ruling out the possibility that the inhibitor is being degraded during the time course of the assay.

In the absence of irreversible inhibition, the biphasic time course observed can be explained only by slow binding inhibition. Preincubation experiments in the presence or absence of substrates showed that inhibition is not substrate-dependent, indicating that mureidomycin A interacts with the free enzyme. Km and Vmax parameters were measured for fluorescent substrate and dodecaprenyl-P in the presence 0, 200, 300, and 400 nM mureidomycin A.2 Km values increased with inhibitor concentration whilst Vmax values were unchanged showing that mureidomycin A is competitive with respect to both substrates (data not shown). In order to calculate the requisite kinetic constants we have assumed a Ping Pong Bi Bi mechanism for the enzyme catalyzed conversion of substrates to products, after the work of Heydanek and et al. (24). We have also assumed, based on the above evidence, that mureidomycin A behaves as a classical dead-end inhibitor (see Fig. 6).

K and K*, equilibrium constants for simple competitive and slow-binding inhibition, were calculated using Equation 1, where v is the initial velocity or steady state final velocity for K or K*, respectively. A, B, and I are the concentrations of dansyl-UDP-MurNAc-pentapeptide, dodecaprenyl-P, and mureidomycin A, respectively.

$$K_{\text{diss}} = \frac{1}{v} = \frac{1}{v_{\text{max}}} + \frac{K_{\text{diss}}}{v_{\text{max}}} + \frac{K_{\text{diss}}}{v_{\text{max}}} \cdot (K_{\text{diss}}/A \cdot v_{\text{max}}) \cdot (K_{\text{diss}}/B \cdot v_{\text{max}}) \cdot (K_{\text{diss}}/C \cdot v_{\text{max}}) \cdot (v_{\text{obs}})}{1} \quad \text{(Eq. 1)}$$

Plots of 1/v versus I yielded values of 36 ± 6 nM and 2.0 ± 0.6 nM for K and K*, respectively (see Fig. 7). In order to measure K and subsequently Kdiss values over a wide range of inhibitor concentrations, it was necessary to record time courses of inhibition at three different enzyme concentrations, since the inherent nonlinearity of the assay time course imposes a time limit for the observation of the onset of inhibition at any given enzyme concentration. Diluted enzyme remains linear over an increase of ~2.5 fluorescence units. More experimental error is observed in the K* data compared to the Km data due to the fact that K* is calculated from the much lower steady state final rates, rather than initial rates which can be measured with a high degree of confidence. However, the observed data is consistent with the slow binding inhibition model, and a value of 2.0 ± 0.6 nM can be deduced for K*.

Values of Kdiss, the observed rate constant for the approach to the steady-state final rate, were determined at each inhibitor concentration using Equation 2 (29), where v is the velocity at time t, and v0 and vs are the initial and steady-state velocities.

$$\ln [(v - v_{\text{obs}})/(v_{0} - v_{s})] = -k_{\text{diss}} \cdot t \quad \text{(Eq. 2)}$$

**Fig. 7.** Determination of slow-binding inhibition kinetic constants. Panel A, determination of K from initial rates in the presence of varying [I]. K was determined from the slope as described in Equation 1. Panel B, determination of K* from final steady-state rates in the presence of varying [I]. K* was determined from the slope as described in Equation 1. Results comprise three sets of data, each at different enzyme concentrations. Consequently, rates are expressed as a percentage of that measured in the absence of inhibitor. Assay mixtures contained 105 μM dansyl-UDP-MurNAc-pentapeptide, 40 μM dodecaprenyl phosphate, and 100 μg/ml phosphatidylglycerol.
The rate constant for the isomerization of E1 to E1*, k_{eq}, was determined by plotting k_{cap} against a function of inhibitor concentration, I, as in Equation 3 (Fig. 8) (29).

\[ k_{\text{obs}} = k_{\text{cat}} + k_{\text{cap}}\left(\frac{1}{(1 + (A/K_{\text{M}}]])^2}\right) \quad \text{(Eq. 3)} \]

From the gradient of this plot, the value obtained for k_{eq} was 0.92 ± 0.2 min^{-1}, whose magnitude is consistent with the time course of the original progress curves.

Attempts were made to measure the rate constant for the dissociation of the tightly bound E-I* complex, k_{diss}, experimentally by rapid separation of enzyme from inhibitor by the method of Penefsky (30). An aliquot of enzyme was inactivated with 0.4 μM mureidomycin A under the assay conditions described under “Experimental Procedures.” The desalted enzyme was completely inactive, but no time-dependent regain of activity was observed. It is likely that the mureidomycin associates with the detergent micelles and so is not effectively separated from the enzyme.

**DISCUSSION**

In order to investigate in detail the molecular basis for inhibition of translocase I by mureidomycin A we have overexpressed and solubilized the E. coli enzyme activity, and have developed a reproducible continuous assay for this enzyme. It is not known why the level of overexpression (28-fold) is so modest given the well documented strength of the trc promoter.

Mureidomycin A was found to inhibit the solubilized enzyme with IC_{50} < 100 nM using a stopped radiochemical assay. Using the continuous fluorescence enhancement assay we have identified mureidomycin A as a slow binding enzyme inhibitor. This provides the first detailed insight into the molecular mechanism of action of this antibiotic. It joins a select group of slow binding enzyme inhibitors which include other examples in peptidoglycan biosynthesis such as the inhibition of β-alanine: β-alanine ligase by aminoalkylphosphinates transition state analogues (31). This mode of enzyme inhibition results from the reversibility of the initial E-I complex into a more tightly binding E-I* complex. In some cases formation of the E-I* complex is due to a conformational change of the enzyme or inhibitor; in other cases it is due to a reversible chemical reaction taking place at the enzyme active site (29). The observation that mureidomycin A is competitive with respect to both dansyl-UDP-MurNAC-pentapeptide and dodecaprenyl-P implies that it acts as a bifunctional inhibitor in the formation of the E-I complex. However, the nature of the transition to the E-I* complex remains to be determined. Studies are in progress to determine the catalytic mechanism of translocase I and the role of catalytic active site residues which may also participate in the mechanism of slow binding inhibition by mureidomycin A.

The K_{m} value of 36 nM determined for mureidomycin A is a remarkable 500-fold lower than the K_{m} of the substrate analogue dansyl-UDP-MurNAC-pentapeptide, and the K_{a} a further 20-fold lower. This binding affinity offers an explanation for its antibacterial potency in vivo, and coupled with an apparent lack of toxicity in mammalian systems means that the elucidation of the mechanism of slow binding inhibition by mureidomycin A could be a basis for the rational design of novel antibacterial agents.

**REFERENCES**

1. Bugg, T. D. H., and Walsh, C. T. (1992) Nat. Prod. Rep. 9, 199–215
2. Swartz, M. N. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2420–2427
3. Neu, H. C. (1992) Science 257, 1054–1073
4. Struve, W. G., Sinha, R. K., and Neuhaus, F. C. (1966) Biochemistry 5, 82–93
5. Higashi, Y., Strominger, J. L., and Sweeney, C. C. (1967) Proc. Natl. Acad. Sci. U.S.A. 57, 1878–1884
6. Ikeda, M., Wachi, M., Jung, H. K., Ishino, F., and Matsuhashi, M. (1991) J. Bacteriol. 173, 1021–1026
7. Bugg, T. D. H., and Brandish, P. E. (1994) FEBS Microbiol. Lett. 119, 255–262
8. Tamura, G., Sasaki, T., Matsuhashi, M., Takakasi, A., and Yanasaka, M. (1976) Agric. Biol. Chem. 40, 447–449
9. Tanaka, H., Oiwa, R., Matsuura, S., and Oumura, S. (1979) Biochem. Biophys. Res. Commun. 86, 906–908
10. Banerjee, D. K. (1989) J. Biol. Chem. 264, 2024–2028
11. Inukai, M., Isono, F., and Takatsuki, A. (1993) Antimicrob. Agents Chemother. 37, 980–983
12. Kimura, K., Miyata, N., Kawanishi, G., Kario, Y., Izaki, K., and Isono, K. (1989) Agric. Biol. Chem. 53, 1811–1815
13. Uzukata, M., and Isono, K. (1988) J. Am. Chem. Soc. 110, 4416–4417
14. Isono, F., Inukai, M., Takakasi, S., Haneshi, T., Kinoshita, T., and Kurowano, H. (1989) J. Antibiot. 42, 677–679
15. Isono, F., Katayama, T., Inukai, M., and Haneshi, T. (1989) J. Antibiot. 42, 674–679
16. Inukai, M., Isono, F., Takakasi, S., Enokiya, R., Sakaida, Y., and Haneshi, T. (1989) J. Antibiot. 42, 662–666
17. Smith, P. K., Krohn, R. I., Herrmann, G. T., Hallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, K., Goek, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–85
18. Kohara, Y., Akiyama, K., and Isono, K. (1987) Cell 50, 495–508
19. Luchtengberg, E. J., and de Haas-Menger, L., and Ryuters, W. H. M. (1972) J. Bacteriol. 109, 926–938
20. Flouret, B., Mengin-Lecreulx, D., and van Heijenoort, J. (1981) Anal. Biochem. 114, 59–66
21. Weisner, W. A., and Neuhaus, F. C. (1977) J. Biol. Chem. 252, 2296–2303
22. Heydanek, M. G., and Neuhaus, F. C. (1969) Biochemistry 8, 1474–1481
23. Verna, A. K., Raizada, M. K., and Schutzbach, J. S. (1977) J. Biol. Chem. 252, 7235–7242
24. Heydanek, M. G., Struve, W. G., and Neuhaus, F. C. (1969) Biochemistry 8, 1214–1221
25. Heydanek, M. G., Jr., Linzer, R., Pless, D. D., and Neuhaus, F. C. (1970) Biochemistry 9, 3618–3623
26. Kaushal, G. P., and Elbein, A. D. (1985) Anal. Biochem. 147, 201–301
27. Pless, D. D., and Neuhaus, F. C. (1973) J. Biol. Chem. 248, 1568–1576
28. Isono, F., Kodama, K., and Inukai, M. (1992) Antimicrob. Agents Chemother. 36, 1024–1027
29. Morrison, J. F., and Walsh, C. T. (1987) Adv. Enzymol. Relat. Areas Mol. Biol. 57, 201–301
30. Penefsky, H. S. (1979) Methods Enzymol. 56, 527–531
31. Duncan, K., and Walsh, C. T. (1988) Biochemistry 27, 3709–3714
32. Segel, I. H. (1993) Enzyme Kinetics, pp. 606–612. Wiley-Interscience, New York
Slow Binding Inhibition of Phospho-N-acetylmuramyl-pentapeptide-translocase (Escherichia coli) by Mureidomycin A
Philip E. Brandish, Martin K. Burnham, John T. Lonsdale, Robert Southgate, Masatoshi Inukai and Timothy D. H. Bugg

J. Biol. Chem. 1996, 271:7609-7614.
doi: 10.1074/jbc.271.13.7609

Access the most updated version of this article at http://www.jbc.org/content/271/13/7609

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 12 of which can be accessed free at http://www.jbc.org/content/271/13/7609.full.html#ref-list-1