Interactions of Soluble Penicillin-Binding Protein 2a of Methicillin-Resistant *Staphylococcus aureus* with Moenomycin†

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ABSTRACT: Kinetics studies in homogeneous aqueous solution showed that solubilized penicillin-binding protein 2a (sPBP2a) of methicillin-resistant *Staphylococcus aureus* (a bacterial DD-peptidase) was inhibited by the amphiphilic glycolipid antibiotic moenomycin. Inhibition at the peptidase site was determined by competition experiments between moenomycin and the chromophoric β-lactam nitrocefin. Under conditions of high salt concentration (1 M NaCl), pseudo-first-order rate constants for the reaction of moenomycin with sPBP2a leading to inhibition of acylation by nitrocefin varied with moenomycin concentration in a biphasic fashion. At low moenomycin concentration (<20 μM) little inhibition occurred, but at higher concentrations a linear increase in rate constant with moenomycin concentration was observed, yielding a second-order rate constant of inhibition of 120 s^{-1} M^{-1}. Since the cmc of moenomycin under these conditions was shown to be ca. 20 μM, the inhibition was concluded to arise from reaction of sPBP2a with a moenomycin micelle. Protein fluorescence studies showed a pseudo-first-order decrease in fluorescence on reaction of the protein with moenomycin. The variation of this rate constant with moenomycin concentration was consistent with reaction of a moenomycin monomer with the protein with a second-order rate constant of 650 s^{-1} M^{-1}. This monomer reaction did not occur at the DD-peptidase site since its rate was unaffected by prior acylation of the enzyme by benzylpenicillin; nor did it inhibit reaction at that site by β-lactams. Under low salt conditions (0.175 M NaCl) where reaction could be studied over a greater range of monomer concentrations since the cmc was ca. 120 μM, similar reactions were involved. Under these circumstances, inhibition was concerted with the reaction of moenomycin monomers, although fast premicellar aggregation of moenomycin with the protein also occurred. All moenomycin interactions with sPBP2a were reversible, as revealed by detergent-extraction chromatography. Lower limits to moenomycin off-rates and equilibrium dissociation constants were 7.7×10^{-4} s^{-1} and 1.2 μM, respectively. Other amphiphiles did not react in exactly the same manner as moenomycin, indicating some degree of specificity in reactions of the latter. sPBP2a did not have detectable affinity for lipid surfaces (Triton X-114 and phosphatidylglycerol vesicles). A general scheme for reaction of moenomycin with sPBP2a is proposed.

The bacterial DD-peptidases or penicillin-binding proteins (PBPs)[1] are the targets of the β-lactam antibiotics (*1*), which are still our best weapon against bacterial infections. These enzymes, which catalyze the last steps of peptidoglycan (bacterial cell wall) biosynthesis, are located on the outer leaf of the bacterial cell membrane. The high molecular weight PBPs have recently been classified by Ghuysen into two groups, based on amino acid sequence, molecular weight, and function (*2*, *3*). Class A enzymes, of molecular weights around 90 K, include PBP1a and PBP1b of *Escherichia coli*, and are known to catalyze both the transglycosylase and transpeptidase reactions of peptidoglycan synthesis. It is the latter of these reactions of course that is specifically inhibited by β-lactam antibiotics. Class B PBPs have molecular weights around 70 K and include PBP2 and PBP3 of *E. coli* as archetypal examples.

As these PBPs are membrane-bound in vivo and occur only in small quantities (ca. 100 copies/cell in *E. coli* for class A and B enzymes (*4*)), they have been difficult to study in detail, both structurally and functionally. In recent years however, water-solubilized constructs, lacking N-terminal membrane anchors, have proved to be useful for molecular studies. Many of the *E. coli* PBPs have been thus solubilized for example. A 3.5 Å resolution crystal structure is available for one such enzyme, PBP2x of *Streptococcus pneumoniae* (*5*).

One medically important class B PBP is PBP2a of *Staphylococcus aureus*. This enzyme is resistant to most of the currently employed β-lactam antibiotics and is the primary determinant of MRSA (methicillin-resistant *S. aureus*) (*6*). Wu et al. (*7*) and, independently, Frank et al. (*8*) have created a solubilized version of this enzyme (sPBP2a) by deletion of a 22 amino acid sequence from the N-terminus. We have studied the reactivity and specificity of sPBP2a against a series of β-lactams and potential acyclic

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[1] Abbreviations: PBP, penicillin-binding protein; sPBP2a, solubilized penicillin-binding protein 2a of *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*; ANS, 8-anilino-1-naphthalenesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; cmc, critical micelle concentration.

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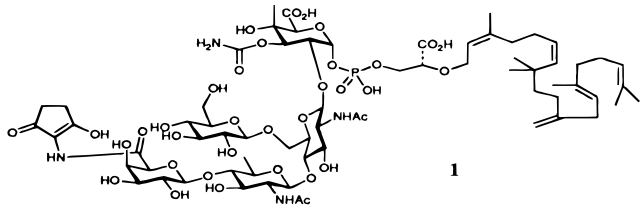
substrates and inhibitors (*9*). Toney et al. (*10*) have described application of a filter-binding assay to obtain the binding constants of several β-lactams to sPBP2a.

Moenomycin, **1**, is an inhibitor of the final transglycosylase reaction of bacterial peptidoglycan biosynthesis (*11*). It has



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been shown to inhibit class A PBPs and, in particular, PBP1a and PBP1b of *E. coli* (*12*, *13*), probably as an intermediate or transition state analogue (*14*). Its specificity for the enzyme is thought to reside in the sugar residues (*14*, *15*) while the hydrocarbon tail may hold the antibiotic in place in the bacterial membrane (*16*), as is thought to occur with the peptidoglycan monomer unit, one substrate of the transglycosylation reaction. Although the preponderance of recent evidence (*2*, *3*, *17*) suggests that class B PBPs do not catalyze the transglycosylase reaction, they must, as DD-peptidases, specifically interact with peptidoglycan structural elements and, presumably, analogues thereof. We describe in this paper our observations of the interactions between moenomycin and sPBP2a of MRSA. This is of specific interest and also as a general model study of interactions between a solubilized PBP and a membrane-directed ligand.

EXPERIMENTAL PROCEDURES

Materials. The mecA (pET11d) expression plasmid was generously supplied by Merck Research Laboratories, Rahway, NJ. Moenomycin was a kind gift from Hoecht Marion Roussel, Frankfurt am Main, Germany. Nitrocefin was purchased from UniPath. Ultrapure sodium dodecyl sulfate (SDS) was purchased from American Bioanalytical. A fluorescent phospholipid, diacyl *N*-(5-(dimethylamino)naphthalenyl-1-sulfonyl)-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (*N*-dansylphosphatidylethanolamine), was obtained from Molecular Probes. The fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS) was obtained from Aldrich, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol), sodium salt (PG), from Avanti Polar Lipids. Benzylpenicillin, L-α-lysophosphatidyl-DL-glycerol palmitoyl (LysoPG), L-α-lysophosphatidylethanolamine palmitoyl (LysoPE), bacteriorhodopsin, bovine serum albumin (BSA), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and Triton X-114 were purchased from Sigma. Sodium deoxycholate was from Schwarz/Mann. The 7.5% SDS-Tris denaturing polyacrylamide gels were purchased from Bio-Rad laboratories. Extracti-Gel D resin was purchased from Pierce.

Concentrated nitrocefin stock solutions were made as previously described (*9*) and the concentrations determined using a previously established extinction coefficient at 386 nm (20 370 M^{-1} cm^{-1} (*18*)). Stock solutions of LysoPE were prepared in 90% ethanol. Stock solutions of all other compounds were made up fresh in reaction buffer. The concentrations of moenomycin stocks were determined using an extinction coefficient of 26 600 M^{-1} cm^{-1} at 259 nm (*19*).

Purification of sPBP2a. The mecA (pET11d) T7 polymerase expression plasmid harboring the sPBP2a gene, from which the N-terminal 22 amino acids containing the putative membrane anchor was removed, has been described previously (*8*). sPBP2a was overexpressed in *Escherichia coli* transformed with the mecA (pET11d) plasmid and purified by ion exchange and gel filtration chromatography (*8*, *9*). The concentration of sPBP2a was determined by measuring the absorbance at 280 nm and using a previously established (*8*) extinction coefficient (81 290 cm^{-1} M^{-1}). The final sample of sPBP2a was greater than 95% pure as judged by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and Coomassie blue staining. Contamination by β-lactamase was determined to be insignificant as indicated by the turnover rate of benzylpenicillin. Stocks of sPBP2a were stored at 4 °C in 25 mM HEPES, pH 7, 1 M NaCl, and 0.01% sodium azide.

Vesicle Preparation. Large unilamellar vesicles of PG, either alone or in the presence of moenomycin, and, in the case of fluorescence experiments, *N*-dansylphosphatidylethanolamine were prepared as follows. Dry samples were mixed and dissolved in 0.2−0.6 mL of methanol. Samples were then vacuum-dried as thin films in small centrifuge tubes. The thin lipid film was resuspended in 25 mM HEPES buffer, pH 7.0, containing 0.175 M NaCl and extruded 29 times through a 100 nm membrane using the LipoFast-Basic membrane extruder (Avestin) according to the manufacturer's instructions. Stock vesicle solutions prepared for the inhibition experiments were generally 30 mM lipid and 0.145 M−1.45 mM moenomycin. Final concentrations of 2 mM lipid and 0.01−0.1 mM moenomycin were used in each experiment.

These solutions containing large unilamellar vesicles were too turbid for fluorescence experiments. Therefore, small unilamellar vesicles were prepared from the large vesicle solutions by sonication on ice using a probe sonicator. Stock vesicle solutions made for the fluorescence experiments were generally 110−84 mM lipid, 1% fluorescent lipid, and 3.5% moenomycin. Final concentrations of 1 mM lipid, 0.01 mM fluorescent lipid, and 0.035 mM moenomycin were used in each experiment. Samples containing the fluorescent lipid were excited at 345 nm for direct fluorescence measurements (emission maximum 520 nm) or at 288 nm for energy transfer measurements. Emission wavelengths of 460−600 nm were monitored.

Determination of Spectrophotometric Kinetic Parameters. Absorption spectra and spectrophotometric reaction rates were measured by means of Perkin-Elmer Lambda 4B and Hewlett-Packard 8452A spectrophotometers. Unless stated otherwise, all reactions described in this paper were performed at 37 °C in a volume of 0.1 mL in 25 mM HEPES buffer, pH 7.0, and either 0.175 or 1.0 M NaCl. Stock solutions of nitrocefin were made fresh as previously described (*9*). Stock solutions of moenomycin, LysoPG, and SDS were made up in reaction buffer and stored at −20 °C.

The rate of reaction between sPBP2a and nitrocefin was determined by monitoring the change in the absorbance of nitrocefin at 482 nm upon acylation of sPBP2a ($\Delta\epsilon$ at 482 nm = 16 000 cm^{-1} M^{-1}). The second-order rate constant for acylation by nitrocefin (k_n) was taken to be 53.2 s^{-1} M^{-1} (*9*). The pseudo-first-order rate constants for the reaction between sPBP2a and moenomycin (k_{M*}) were measured by

Scheme 1

$$E + N \xrightarrow{k_n} EN$$
$$E + M \xrightarrow{k_M} EM$$
$$E + N \xrightarrow{k_o} E + P$$

direct competition with nitrocefin for sPBP2a (Scheme 1).

The change in absorbance at 482 nm with time was fitted to eq 1, where k_{n*} is the pseudo-first-order rate constant for

$$A = A_0 + \epsilon_n E_0[k_{n*}/(k_{n*} + k_{M*})][1 - e^{-(k_{n*} + k_{M*})t}] + k_0 N_0 E_n E_0 t \quad (1)$$

acylation by nitrocefin at the concentration N_0 (20−200 μM) employed, k_{M*} is the pseudo-first-order rate constant for the reaction of sPBP2a with moenomycin, E_0 is the initial enzyme concentration (1−3 μM), and k_0 is the second-order rate constant for the nonspecific enzyme-catalyzed hydrolysis of nitrocefin (*9*). The second-order rate constant (k_M) for reaction between sPBP2a and moenomycin in 1.0 M NaCl was calculated by fitting the kinetic data to eq 2, where M_0

$$k_{M*} = k_M(M_0 - \text{cmc}) \quad (2)$$

is the concentration of total moenomycin employed and the critical micelle concentration (cmc) was taken to be is 20 μM (see below). The second-order rate constant for the reaction between sPBP2a and moenomycin in 0.175 M NaCl was calculated directly (see below).

The reactions between sPBP2a and lysoPG or other amphiphiles were also monitored by direct competition with nitrocefin as described above.

Determination of Spectrofluorimetric Kinetic Parameters. Spectrofluorometric measurements were performed using a Perkin-Elmer MPF 44A spectrofluorimeter in cuvettes holding either 0.1 or 0.4 mL reaction volumes. The pseudo-first-order rate constants for the reaction between sPBP2a and moenomycin or the lysophospholipids were measured directly by monitoring changes in the fluorescence of sPBP2a. The intrinsic fluorescence of sPBP2a was determined by exciting samples at 288 nm and measuring the emission at 335 nm as previously described (*9*). Changes in fluorescence due to the interaction with moenomycin were measured during reaction of sPBP2a (1 μM) with various concentrations of moenomycin (0−400 μM). Apparent first-order rate constants for the decrease in fluorescence were calculated by fitting the change in the relative fluorescence with time to a single-exponential equation. A second-order fitting procedure was employed for moenomycin concentrations below 10 μM.

Fluorescence Energy Transfer Experiments. The 0.1 mL samples of small unilamellar PG vesicles containing the fluorescent lipid (1%) and in some cases moenomycin (up to 3.5%) were excited at 288 nm in the presence of 1 μM sPBP2a, 25 mM HEPES, pH 7.0, and 1 M NaCl at 37 °C. The emission was monitored from 460 to 600 nm and the maximum at 520 nm followed as a function of time.

Determination of Critical Micelle Concentrations. All critical micelle concentrations (cmcs) were measured by enhancement of the fluorescence of the anionic probe ANS (*20*, *21*). Fluorescence spectra and intensities of ANS were obtained at 37 °C at an excitation wavelength of 370 nm and emission wavelengths of 460−600 nm. Fluorescence titrations were performed by the addition of small aliquots of the amphiphile solution to a solution of ANS (1 μM or 10 μM) in 25 mM HEPES buffer, pH 7, and either 0.175 or 1.0M NaCl. Corrections were made for dilution of the ANS probe. Maximal emission intensities were plotted versus the concentration of surfactant, and two straight lines were drawn through the points. The cmc for each surfactant was taken to be the concentration at which the two lines intersected. The emission intensity change upon addition of each surfactant solution was shown not to be time dependent.

Triton X-114 Phase Partitioning of sPBP2a. The detergent binding properties of sPBP2a were assessed by Triton X-114 phase partitioning, as previously described for integral membrane proteins by Bordier (*22*) and for *E. coli* PBP1b by Wang et al. (*23*). Protein samples (2−4 μM) were prepared on ice in 200 μL of 25 mM HEPES, pH 7, 0.175 M NaCl, and 1% Triton X-114. A cushion (300 μL) of 6% (w/v) sucrose, 25 mM HEPES, pH 7, 0.175 M NaCl, and 0.06% Triton X-114 was placed in a 1.5 mL plastic centrifuge tube. The clear protein sample was placed on top of the cushion and the resulting suspension incubated for 5 min at 37 °C. The cloudy solution was centrifuged at 300*g* at room temperature, and the upper aqueous phase was removed to a new tube and received another aliquot (5 μL) of 20% Triton X-114. The mixture was reclarified on ice for 5 min and then again overlaid on the same sucrose cushion, incubated 5 min at 37 °C, and recentrifuged for 5 min. The aqueous (detergent-poor) phase was then placed in a clean tube, given a final rinse with 2% Triton X-114, and recentrifuged without a cushion. Protein from both the detergent-rich and detergent-poor samples was precipitated with 7 volumes of acetone on ice for 30 min and collected by means of a 15 min centrifugation (12 000 rpm) at room temperature. These samples along with the final wash pellet were dissolved in 30 μL of SDS-sample buffer (12.5 mM Tris, pH 7, 0.5% SDS, 2.5% glycerol, 0.005% bromophenol blue, 0.25 mM β-mercaptoethanol), boiled for 5 min, and analyzed together with an input protein sample on a 7.5% SDS−PAGE gel and, after development, stained with Coomassie blue.

Removal of Moenomycin by an Extracti-Gel D Column. A 215 μL sample of 4.0 μM sPBP2a in 25 mM HEPES, pH 7, and 1 M NaCl was incubated at 37 °C with 100 μM moenomycin. An 80 μL sample was removed after 30 min and checked for inactivation at 37 °C by addition of 20 μL of nitrocefin (final concentration of 50 μM) as described above. The remaining 135 μL of sample was applied to a 1.0 mL Extracti-Gel D detergent-removing column equilibrated with 25 mM HEPES, pH 7, and 1 M NaCl at room temperature. The protein was eluted from the column in the same buffer in 100−120 μL fractions. The concentrations of moenomycin and of protein in each fraction were determined from the absorbance at 259 and 280 nm and the extinction coefficients for moenomycin and sPBP2a at those wavelengths. The quantity of moenomycin which was not absorbed by the column was determined by running 135 μL of 100 μM moenomycin on a separate column. The activity of the sPBP2a in the peak fractions was determined by measuring the rate of reaction with 50 μM nitrocefin at 37 °C as described above.

RESULTS AND DISCUSSION

As will become clear from the results described below and the subsequent discussion, the reaction between sPBP2a and moenomycin is complicated by the tendency of the latter, with its detergent-like amphiphilic structure **1**, to aggregate in aqueous solution. This aggregation has been previously observed but not studied in detail. Light scattering experiments (*19*) indicated a particle weight in aqueous solution at pH 7 of up to 70 000 and thus an aggregate, presumably a micelle, of some 40 moenomycin monomers. Since moenomycin is an anionic amphiphile, its cmc is strongly dependent on salt concentration (*24*). In this work we have employed two concentrations of sodium chloride, 0.175 M ("low salt" henceforward) and 1.0 M ("high salt") in order to more effectively study the reaction of moenomycin with sPBP2a both below and above the moenomycin cmc. We have attempted to merge the results from these two regimes into a general picture of the moenomycin/sPBP2a interaction.

At high salt concentration, moenomycin appeared to competitively and irreversibly inhibit acylation of the DD-peptidase site of sPBP2a by nitrocefin. Application of Scheme 1 to the spectrophotometric data (Figure 1A) led to the relationship between the pseudo-first-order rate constant of reaction of moenomycin with sPBP2a and the moenomycin concentration shown in Figure 1B. Two phases of reaction appear to occur. At moenomycin concentrations below about 20 μM, the rate of inhibition is very small, while above this concentration the rate appears to increase linearly with moenomycin concentration. The apparent second-order rate constant from the linear phase was (118 $\pm$ 25) s^{-1} M^{-1}.

The likely source of the discontinuity evident in Figure 1B is found in Figure 1C, which shows the fluorescence intensity of ANS (10 μM) as a function of moenomycin concentration. Again a discontinuity at moenomycin concentration of about 20 μM is observed, one that in this case can be interpreted as due to cooperative micelle formation with a cmc of 20 μM. The same result was obtained if 1 μM ANS was employed. Thus, inhibition by moenomycin of the acylation of sPBP2a by nitrocefin likely involves interaction of each sPBP2a molecule with a moenomycin micelle. Note that the absorption spectrum of nitrocefin was not affected by moenomycin at the concentrations employed in the experiments shown in Figure 1 and thus sequestration of nitrocefin by moenomycin micelles is unlikely to explain the results. [The absorption spectrum of nitrocefin is medium (solvent) sensitive; for example, in ethanol and acetonitrile, less polar solvents than water, as would be the interior of a micelle, blue shifts of 6 and 11 nm, respectively, were observed.] A reduction of effective nitrocefin concentration by sequestration in moenomycin micelles would also lead to smaller pseudo-first-order rate constants of reaction of nitrocefin with sPBP2a rather than larger as observed (Figure 1A,B) and as required by Scheme 1. The inhibition reaction was unaffected by the presence of Mg^{2+} (5 mM).

Further insight into the interaction of moenomycin with sPBP2a is afforded by an examination of the effect of moenomycin on protein fluorescence. As seen in Figure 2A, addition of moenomycin to sPBP2a led to a first-order time-dependent decrease in protein fluorescence. First order rate constants for this process as a function of moenomycin

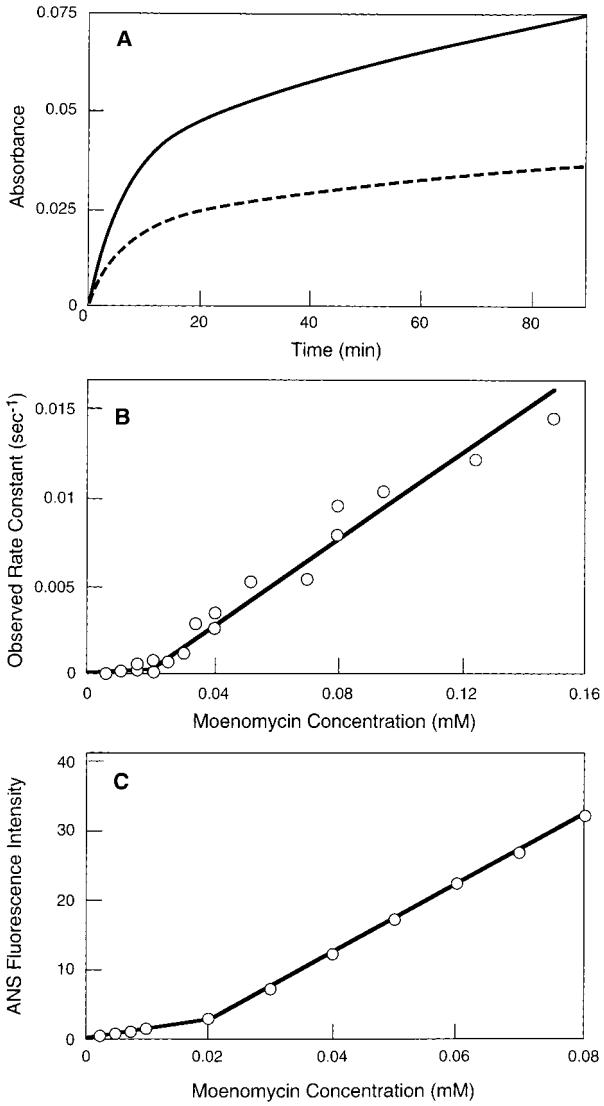


FIGURE 1: (A) Absorbance changes at 482 nm as a function of time on reaction of sPBP2a (2.2 μM) with nitrocefin (50 μM) in the absence (solid line) and presence (dashed line) of moenomycin (40 μM). (B) Observed pseudo-first-order rate constants for reaction of sPBP2a (2 μM) with moenomycin, as measured by competition with nitrocefin (50 μM). (C) Fluorescence emission intensity changes at 495 nm (excitation at 370 nm) on titration of ANS (10 μM) with moenomycin. The above experiments were conducted in 1.0 M NaCl.

concentration are shown in Figure 2B. It is noticeable that these rate constants are greater than the inhibition rate constants of Figure 1 and do not increase linearly with moenomycin concentration but rather increase in a hyperbolic-like manner with apparent saturation above 0.4 mM moenomycin. These differences clearly indicate the presence of a different mode of interaction of moenomycin with sPBP2a than that described above.

Consideration of Figure 2B and Figure 1C together suggests that the change in protein fluorescence represented in Figure 2A arises from reaction of sPBP2a, not with a moenomycin micelle but with a monomer. Some reaction with a small oligomer is also possible (see below). The rate of reaction with a monomer would be expected to increase linearly with concentration below the cmc, but the slope would then decrease to zero above the cmc, as observed (Figure 2B). The concentration of monomers is believed to

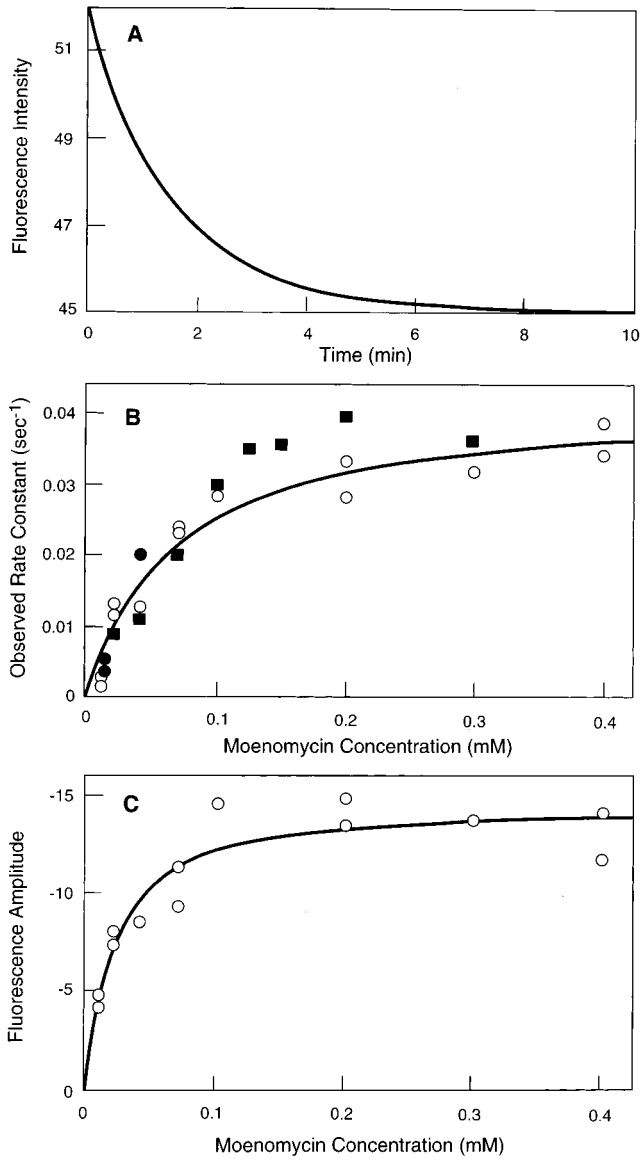
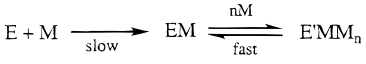


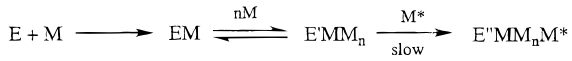
FIGURE 2: (A) Protein fluorescence emission change at 335 nm (excitation at 288 nm) as a function of time on reaction of sPBP2a (1 μM) with moenomycin (40 μM). (B) Observed pseudo-first-order rate constants for reaction of sPBP2a (1 μM) with moenomycin as determined from protein fluorescence changes (A) in the absence of benzylpenicillin (○) and after acylation of sPBP2a by 100 μM benzylpenicillin (■). Also shown (●) are rate constants measured by changes in ANS (10 μM) fluorescence emission. The solid line is a hyperbola fitted to the protein fluorescence data (○) (see text). (C) Amplitude of the protein fluorescence emission changes on reaction of sPBP2a (1 μM) with moenomycin. The solid line is a hyperbola (see text). The above experiments were conducted in 1.0 M NaCl.

remain close to constant above the cmc while that of micelles will increase linearly (*24*). The second-order rate constant for reaction of a monomer with sPBP2a would then be (650 ± 130) s^{-1} M^{-1}. This value is taken from the slope at zero moenomycin concentration of a hyperbola fitted by a nonlinear least-squares procedure to the fluorescence data of Figure 2A. The same fitting procedure yields a value of 0.041 s^{-1} at the apparent saturation level. If this reaction involved only monomers, a second-order rate constant should also be obtained by division of this plateau value by the cmc. This procedure yields a second-order rate constant of 2×10^3 s^{-1} M^{-1}. This value is clearly greater than that obtained from the initial slope and may reflect the presence of some dimer or oligomer reaction.

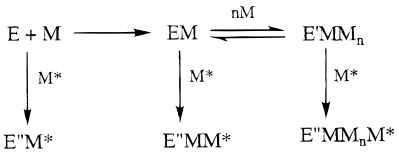
Scheme 2



Scheme 3



Scheme 4



The reaction leading to the protein fluorescence change of Figure 2A has further complications however. Figure 2C shows the amplitude of the fluorescence change is a function of moenomycin concentration. This result is inconsistent with a reaction scheme composed of a simple one-step irreversible reaction or an irreversible step preceded by one or more equilibria. Also inconsistent with the data is a one step monomer binding equilibrium since saturation in monomer binding is not possible above the cmc where the concentration of monomers does not change. The data of Figure 2B,C therefore seem best accommodated by Scheme 2, where the binding of a monomer M leads to a complex EM, which promotes fast equilibrium aggregation of moenomycin on the protein. The latter process, perhaps by means of a protein conformational change (E → E′), is accompanied by partial quenching of protein fluorescence. It saturates as micelles become the dominant form of moenomycin above the cmc (20 μM).

It is necessary also to relate the slower inhibition reaction, shown to involve moenomycin micelles, with Scheme 2. This can be done either by means of a linear sequence (Scheme 3), where the inactive (toward acylation by nitrocefin) enzyme, E″MM_nM*, arises directly from reaction of E′MM_n with a moenomycin micelle, M*, or in a branched, noncompetitive fashion (Scheme 4), where the reactions at low moenomycin concentration and the inhibition are completely unrelated. As discussed below, Scheme 3 may be the better model. The distinction between the second and third steps of Scheme 3 is demonstrated by the fact that addition of benzylpenicillin to a reaction mixture after 4.5 half-times of the protein fluorescence quenching reaction gives rise to a fluorescence increase from the acylation reaction (*9*). An almost full burst of reaction with nitrocefin is also observed at this stage. If the two steps were not distinct, no such fluorescence increase on addition of penicillin or absorption change on reaction with nitrocefin would be expected.

In either Scheme, 3 or 4, E, EM and E′MM_n represent enzyme forms that react with nitrocefin at comparable rates. This is also true with benzylpenicillin since the protein fluorescence increase produced by acylation with benzylpenicillin (*9*) can be observed after formation of EM and E′MM_n but prior to reaction with M*. It is striking however that reaction of sPBP2a with benzylpenicillin does not change the rate of the protein fluorescence event on addition of

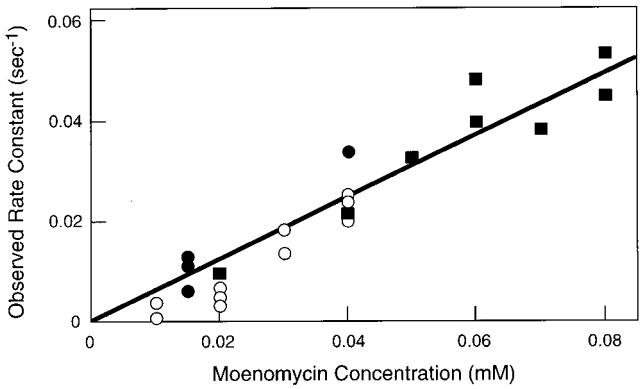


FIGURE 3: Observed pseudo-first-order rate constants for reaction of sPBP2a (2 μM) with moenomycin, as measured by competition with nitrocefin (○) and by protein fluorescence (■). Also shown (●) are rate constants measured from changes in ANS (10 μM) fluorescence. These experiments were conducted in 0.175 M NaCl.

moenomycin (see Figure 2A), although the amplitude of the change is somewhat increased (not shown).

The interpretation of events offered above is supported by observations under the low salt regime. A pseudo-first-order decrease in protein fluorescence was also observed under the latter conditions. A plot of the pseudo-first-order rate constants vs moenomycin concentration is shown as Figure 3. The relationship may well be linear, yielding an apparent second-order rate constant of (630 ± 100) s^{-1} M^{-1}. It is noticeable that this value is very similar to that deduced for reaction of monomeric moenomycin under high salt conditions. It seems likely that the same reaction is involved. The linearity of Figure 3 and the derived moenomycin monomer reactivity correlate well with the fact that the cmc of moenomycin was shown by titration against ANS (data not shown) to be ca. 120 μM at 0.175 M NaCl, i.e., higher than the highest concentration of moenomycin employed in the experiments of Figure 3. Thus, under the experimental conditions of Figure 3, the concentration of moenomycin *monomers* would increase linearly with total moenomycin concentration. These findings at low salt concentration strongly support the proposition that the moenomycin reaction leading to quenching of the protein fluorescence predominantly involves moenomycin monomers.

A feature of the reaction of moenomycin under low salt conditions that at first seems discordant with the high salt results is that the interpretation of the latter seemed to require further moenomycin aggregation to achieve the fluorescence quenching (Scheme 2), where this requirement was evident in the variation of the extent of quenching with moenomycin concentration (Figure 2C). In contrast, the extent of quenching at low salt appeared to be independent of moenomycin concentration and comparable to the maximum change at high salt. The latter suggests that the change in the protein represented by EM → E′MM_n of Scheme 2, and requiring additional moenomycin aggregation, must also have occurred at low salt at moenomycin concentrations between 10 and 80 μM. The presence of such aggregation was tested by incorporation of a probe, ANS, into the system.

Figure 4A shows fluorescence emission spectra of 10 μM ANS in the presence of sPBP2a alone under low salt conditions, immediately after addition of moenomycin (15 or 40 μM) to this mixture, and then again after completion of a time-dependent change in the spectrum. There are

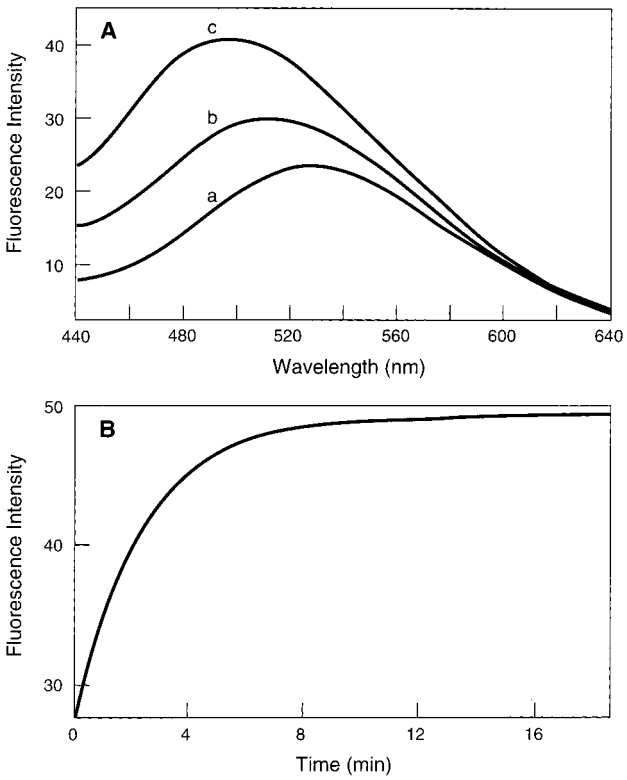


FIGURE 4: (A) Fluorescence emission spectra under low salt conditions (a) of ANS (10 μM) in the presence of sPBP2a (1 μM) and (b) of sample a immediately after addition of moenomycin (15 μM) and (c) after the time-dependent reaction had proceeded to completion. The exitation wavelength was 370 nm. (B) Fluorescence emission changes at 490 nm as a function of time on reaction of sPBP2a (1 μM) with moenomycin (15 μM) in the presence of ANS (10 μM). This represents the time dependence of the spectral change (b) to (c) in (A).

Scheme 5

$$E + nM \underset{}{\overset{\text{fast}}{\rightleftharpoons}} E.M_n + M \xrightarrow{\text{slow}} E'MM_n$$

several points to note here. First, the fluorescence of ANS in solution is not affected by either sPBP2a or by moenomycin below its cmc. Therefore the immediate ANS spectral change on addition of moenomycin to the protein is suggestive of some degree of fast association of moenomycin with the protein to form a complex that binds ANS rapidly, prior to the slower reaction of Figure 3. Second, the subsequent time-dependent enhancement of fluorescence (Figure 4B) is suggestive of a rearrangement of the initial complex, possibly involving protein conformational change and coordinated reorganization, or perhaps further aggregation, of moenomycin. Similar observations were made under high salt conditions. It is important to note that the pseudo-first-order rate constant of the slow step here, when monitored by ANS fluorescence, is the same, within experimental uncertainty, as that observed by protein fluorescence in the absence of ANS (see relevant points in Figures 2B and 3). This result provides evidence that the ANS event is not influenced by the presence of ANS; i.e., ANS is, at this level at least, an inert probe.

The observations described in the last paragraph suggests the scenario of Scheme 5 for the low salt reaction. Resolution of the differences between Schemes 2 and 5 will be addressed below.

Scheme 6

$$E + nM \underset{\text{fast}}{\rightleftharpoons} EM_n + M \xrightarrow[\text{fast}]{} E'MM_n \xrightarrow[\text{slow}]{mM} E'MM_{m+n}$$

Scheme 7

$$E + nM \underset{\text{fast}}{\rightleftharpoons} EM_n + M \xrightarrow[\text{slow}]{} E'MM_n \xrightarrow[\text{fast}]{mM} E'MM_{m+n}$$

Scheme 8

$$E + nM \underset{\text{fast}}{\rightleftharpoons} EM_n + M \xrightarrow{\text{slow}} E'MM_n \underset{\text{fast}}{\overset{mM}{\rightleftharpoons}} E'MM_{m+n}$$

First however, it should be noted that moenomycin also produces a competitive inhibition of acylation of the DD-peptidase site by nitrocefin and benzylpenicillin at low salt just as it did at high and despite the fact that at low salt, with moenomycin concentrations below the cmc, there would be few micelles available—cf. Schemes 3 and 4. The rate constants measured for this inhibition of the nitrocefin reaction are also shown in Figure 3. These appear to indicate a reaction rate somewhat slower than the fluorescence change at low moenomycin concentrations, suggesting the requirement for more moenomycin, but comparable to it at higher concentrations, suggesting that at these concentrations the rate-determining steps of the two processes are the same. This can be represented as a progression from Scheme 6 at low moenomycin concentration, where $E'MM_{m+n}$ represents the enzyme unreactive with β-lactams, to Scheme 7 at higher moenomycin concentrations. It might also be noted that under low salt conditions, in the presence of moenomycin, protein precipitation did not occur after acylation by nitrocefin as it did in the absence of moenomycin (*9*). This observation is also suggestive of significant interaction between moenomycin and sPBP2a.

Scheme 6 is obviously very similar to Scheme 3 which represents the high salt situation except that the further aggregation leading to inhibition in the latter involves the binding of monomers or oligomers while in the latter the binding of a micelle. Noncompetitive slow binding of monomers/oligomers at low salt, analogous to that of micelles at high salt in Scheme 4, might represent an alternative to Scheme 6, but the similarity of the rate constants for fluorescence changes and inhibition at low salt does argue for the linear process, at least under those conditions.

Finally, it should be noted that the presence of fast moenomycin binding under high salt conditions was also demonstrated by the ANS probe. The results were similar to those at low salt. In particular, at high salt, a first-order time-dependent enhancement of ANS fluorescence was also observed, where the rate constants matched those of the protein fluorescence change (see relevant points in Figure 2B). Thus expansion of Scheme 2 to Scheme 8 is necessary. The additional moenomycin (as a micelle) required to effect enzyme inhibition at high salt may reflect the greater stability of the protein under these conditions (*9*). The faster inhibition at low salt (compare Figure 1B with Figure 3) may also reflect this point.

Reversibility of the Moenomycin/sPBP2a Reaction. Although the complex interaction between moenomycin and sPBP2a proceeds to apparently irreversible inhibition of the DD-peptidase site, the interaction between the protein and moenomycin is probably noncovalent and can be reversed. For example, passage of a solution (0.135 mL, high salt) of the moenomycin/sPBP2a complex (containing 0.54 nmol of protein and 13.5 nmol of moenomycin) through an Extracti-Gel D column yielded 89% recovery of 90−100% active (toward acylation by nitrocefin) sPBP2a. The fractions containing the active enzyme contained 0.48 nmol of protein (estimated by absorbance at 280 nm) and 0.29 nmol of moenomycin (estimated by absorbance at 258 nm). In a control experiment without protein, the same fraction contained 0.27 nmol of moenomycin. Thus essentially no moenomycin remained associated with the protein after passage through the detergent-binding column and therefore the binding between moenomycin and sPBP2a and the inhibition reaction must be noncovalent.

Passage of a mixture of sPBP2a (6 μM) and moenomycin (60 μM) through a Sephadex G50 column (14 × 0.5 cm) also led to separation of most of the ligand from the protein. The leading fractions of PBP2a, emerging after ca. 15 min, retained approximately 0.5 moenomycin molecules/sPBP2a. This suggests a minimum off-rate for any bound moenomycin of 7.7×10^{-4} s^{-1}.

Specificity of the Moenomycin Reaction. Reaction of sPBP2a with Amphiphiles. In view of the mechanism of moenomycin interaction with sPBP2a described above, involving aggregation of a significant number of moenomycin molecules with the protein, the issue of specificity arose. Do all amphiphiles react with sPBP2a in this manner? Two long chain (C16) lysophospholipids, lysoPE and lysoPG, were chosen to test since they are known to form micelles (*25*). Further, the headgroups are typical of bacterial membrane lipids (*26*) and the latter compound, like moenomycin, has a negatively charged headgroup. At a concentration of 20 μM (most likely above the cmc in each case (*25*)) neither compound affected the nitrocefin reaction with sPBP2a at either low or high salt. At higher concentration (100 and 345 μM), the lysoPG did tend to inhibit the nitrocefin reaction (3−4-fold) but not competitively and not irreversibly since the amplitude of the nitrocefin reaction increased 2−3-fold. The rate of inhibition of sPBP2a by moenomycin was not affected however. The presence of the phospholipid, unlike moenomycin, did not affect precipitation of the protein after acylation by nitrocefin at low salt (*9*). Under both high and low salt conditions, the lysophospholipid (100 μM) gave rise to a very slow decrease in protein fluorescence with time.

Sodium dodecyl sulfate (5−200 μM, at high salt, where the cmc was determined to be 150 μM by titration against ANS) appeared to cause an increase in the rate of sPBP2a acylation by nitrocefin (3−4-fold) with no effect on the amplitude of the reaction. It subsequently caused precipitation of the protein, independent of the presence of nitrocefin. Sodium deoxycholate (0.48 mM) and the zwitterionic detergent CHAPS (0.32, 1.6 mM), both at concentrations below their respective cmcs, did not affect the rate of the nitrocefin reaction with sPBP2a. CHAPS did not, unlike moenomycin, affect the protein fluorescence.

Although there may well be interactions between sPBP2a and detergents in general, those tested above do not seem to reproduce the characteristics of the moenomycin reaction.

Affinity of sPBP2a for Lipid Surfaces. The affinity of sPBP2a for lipid surfaces was assessed, employing two such surfaces, Triton X-114 micelles and PG vesicles. Triton X-114 at 37 °C separates into two phases, one detergent rich

and the other detergent poor. Water-soluble proteins should partition into the latter, and lipophilic proteins, into the former (*22*, *23*). For example, in our hands, SDS−PAGE analysis showed that essentially all of the soluble protein bovine serum albumin partitioned into the latter phase while the intrinsic membrane protein bacteriorhodopsin was found in the former. Even in 4% Triton X-114, essentially all of sPBP2a was observed (SDS−PAGE electrophoresis) in the detergent-poor phase (data not shown). This suggests that the surface of sPBP2a does not contain a membrane-association region. Such a site has been found by this method on the class A penicillin-binding protein PBP1b of *E. coli* even after removal of the N-terminal transmembrane anchor by proteolysis (*23*). PBP2 of *E. coli* however did not exhibit such a site (*23*).

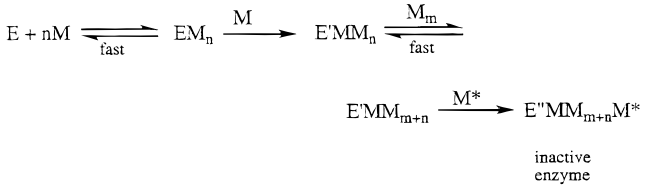
Solutions containing PG vesicles (1.54 mg/mL; 2 mM) were prepared by extrusion in low salt buffer (see Experimental Section), in the presence and absence of 10−100 μM moenomycin. Neither the vesicles alone nor the vesicles containing moenomycin had any effect on the rate or amplitude of the reaction between sPBP2a and nitrocefin. It seems likely from these results that moenomycin was indeed incorporated into the vesicles. Structural studies have demonstrated the affinity of moenomycin for phospholipid bilayers (*27*). The vesicles, with or without moenomycin, had no effect on protein fluorescence.

PG vesicles (1 mM) containing a fluorescent probe, *N*-dansylphosphatidylethanolamine, 10 μM, with and without moenomycin (35 μM) were also prepared. The dansyl fluorescence emission (520 nm) of these preparations was not affected by sPBP2a (1 μM) either at an excitation wavelength of 345 nm or at 288 nm. The former wavelength monitors the environment of the dansyl group directly while the latter was used in order to detect protein to vesicle energy transfer such as would likely occur on protein−vesicle contact (*28*).

These observations provide further evidence that sPBP2a has little general affinity, in the absence of its N-terminal peptide, for lipid surfaces. Further, the results show that moenomycin in a phospholipid bilayer is not available to sPBP2a. This includes availability not only for the interactions that involve aggregation of moenomycin, a result that is perhaps not surprising, but also for the monomer interaction.

An effect produced by CHAPS probably relates to the vesicle results described above. Although, as mentioned above, CHAPS alone had no effect on the reaction of sPBP2a with nitrocefin under high salt conditions, it did, at 0.32 and 1.6 mM, below the cmc (determined by titration against ANS to be 3.5 mM in 1 M NaCl), progressively diminish the extent of inhibition by moenomycin. A titration of moenomycin (0−80 μM) against a mixture of 10 μM ANS with 1.6 mM CHAPS showed the presence of mixed micelles of moenomycin and CHAPS down to at least 5 μM of the former. Apparently sPBP2a is unable to extract moenomycin from this mixed micelle just as it could not from a vesicle. Sodium deoxycholate did not have this effect. It is likely that the negatively charged moenomycin has less tendency to associate with the negatively charged deoxycholate than with the zwitterionic CHAPS. Moenomycin is known to associate with SDS micelles however (*29*).

Scheme 9



General Discussion. The experiments described above lead to Scheme 9 as a conceptual framework for the complex aggregation process of moenomycin binding to sPBP2a, a process gradually building up from monomer or premicelle oligomer interaction with the protein to, at moenomycin concentrations above the cmc, association of the protein with a micelle M*. Although initial moenomycin binding does not appear to affect the DD-peptidase (β-lactam-binding) site, later aggregation, probably following a conformational change to the protein, does disrupt it, effectively irreversibly. Embedded in this sequence of events is one unique event, apparently involving the reaction of a monomeric moenomycin with the protein and which may be seminal to a protein conformational change (characterized by significant quenching of the protein fluorescence) and probably to inhibition of the DD-peptidase site. In this scheme, the binding of *n*M and M may be random rather than sequential and so might, although perhaps less likely, the binding of M*. It is not known just how many separate binding sites for moenomycin might be involved. Although it is reasonable to assume that the unique monomer M might interact at a specific site, the aggregation represented by the binding of M_{m+n} and M* might well occur at a common site or area on the protein surface. It seems that neither of these moenomycin binding regions directly overlaps with the DD-peptidase site, and thus, the inhibition of the β-lactam reaction by moenomycin micelles must arise by indirect means.

It is important to realize that Scheme 9 is meant to be very general, in the sense that it is likely that it will change in quantitative detail with reaction conditions. It is clear from the present work for example that different steps become more or less important depending on the moenomycin concentration relative to the cmc. The latter is of course dependent on the salt concentration. It is also likely that the nature of aggregates, including micelles, of moenomycin changes with salt concentration (*24*), both for moenomycin alone and in its protein complexes.

It is also important to note that many of the elements of Scheme 9 are well-known from studies of the interactions of other proteins with amphiphiles. For example, progressive and often positively cooperative binding of amphiphiles to proteins is well-known (*30*−*33*). This aggregation often begins well below the cmc of the ligand, i.e., as protein-induced aggregation of ligand monomers or premicelles (*34*−*39*). Sparingly water-soluble organic dyes, including ANS, have been found to partition into these complexes (*36*, *40*−*42*). Specific enzyme inhibition by micelles has also been reported (*43*), as is occlusion of inhibitors in mixed micelles (*44*, *45*). The change in conformation of an enzyme by interaction with a micelle (containing a competitive inhibitor) has been documented in detail in the case of phospholipase A2 (*46*−*48*). The binding of amphiphilic inhibitors at more than one site on an enzyme has also been reported (*49*).

The second-order rate constant for reaction of monomeric moenomycin with sPBP2a was found to be ca. 650 s^{-1} M^{-1}. It is noteworthy that the comparable figure for benzylpenicillin was only 12 s^{-1} M^{-1}. The latter value clearly illustrates the resistance of sBP2a to β-lactams (*9*). Combination of the monomer on-rate with the lower limit of the off-rate estimated from Sephadex chromatography leads to a lower limit of the moenomycin equilibrium dissociation constant of 1.2 μM.

Moenomycin is best-known as an inhibitor of the transglycosylase reaction of PBP1b of *E. coli*. This inhibition reaction is apparently complete in less than 30 min at submicromolar moenomycin concentrations, although the time dependence of the inhibition does not seem to have been established (*12*, *13*, *50*). It seems likely therefore that moenomycin reacts more rapidly with PBP1b than with sPBP2a and forms a tighter complex. It should be noted however that the PBP1b reactions were carried out with the holo enzyme, retaining the transmembrane anchor, in solutions containing 0.1% deoxycholate. The effect of moenomycin on solubilized PBP1b (*23*, *51*) does not seem to have been yet reported. The moenomycin/PBP1b interaction is reversible (*13*), just as it is for sPBP2a. It is also known that acylation of the DD-peptidase site of PBP1b by a β-lactam has little effect on transglycosylation rates; with some β-lactams in fact the rate of transglycosylation increases (*11*, *50*). This suggests that the transglycosylase site may also be open to moenomycin in the acylated enzyme, as found in this work with sPBP2a, where acylation by benzylpenicillin did not significantly affect the moenomycin monomer reaction.

Other bacterial enzymes have also been reported to be inhibited by moenomycin and moenomycin analogues although at higher concentrations (*52*−*56*). More importantly perhaps, other enzymes are reported not to be inhibited. In particular, the latter group contains bacterial monofunctional glycosyl transferases which are also able to participate in peptidoglycan synthesis (*57*, *58*).

We have shown that moenomycin reacts readily with sPBP2a in a complex series of reactions involving aggregation of the former at the protein surface. These include the binding of a moenomycin monomer at a site distinct from the DD-peptidase (β-lactam binding) site and inhibition of β-lactam binding by further aggregation steps. The monomer site might represent a yet undiscovered transglycosylase site or, alternatively, either the donor or the acceptor region of the DD-peptidase site. It is difficult to say with certainty whether a moenomycin micelle would be required for inhibition of membrane-bound PBP2a in vivo. It may be that when the enzyme is membrane-bound, a monomer of membrane-bound moenomycin would be sufficient. The micellar inhibition of sPBP2a may be then, to some degree, an artifact of our use of the solubilized protein. The affinity of the protein for moenomycin is such that, at the moenomycin concentration needed to significantly bind to the enzyme, moenomycin micelles, either free in solution or induced by the protein, also enter the picture. If the affinity of the enzyme for moenomycin were slightly greater, it presumably would have been easier to separate monomer from micelle binding. Nonetheless this study should provide a useful model of what will be seen when the interactions of moenomycin with other solubilized PBPs are examined. An important next step to better assess the specificity of the moenomycin structure would involve a study of the reaction of PBPs with delipidomoenomycin (*59*).

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REFERENCES

1. Tipper, D. J., and Strominger, J. L. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1133−1141.
2. Ghuysen, J.-M. (1994) *Trends Microbiol. 2*, 372−380.
3. Ghuysen, J.-M. (1997) *Int. J. Antimicrob. Agents 8*, 45−60.
4. Dougherty, T. J., Kennedy, K., Kessler, R. E., and Pucci, M. J. (1996) *J. Bacteriol. 178*, 6110−6115.
5. Pares, S., Mouz, N., Petillot, Y., Hakenbeck, R., and Dideberg, O. (1996) *Nature Struct. Biol. 3*, 284−289.
6. Maple, P. C., Hamilton-Miller, J.-M., and Brunfit, W. (1989) *Lancet* i, 537−539.
7. Wu, C. Y. E., Hoskins, J., Blaszczak, L. C., Preston, D. A., and Skatrud, P. L. (1992) *Antimicrob. Agents Chemother. 36*, 533−539.
8. Frank, L. J., Wisniewski, D., Hammond, G. G., Hermes, J., Marcy, A., and Cameron, P. M. (1995) *Protein Express. Purif. 6*, 671−678.
9. Graves-Woodward, K., and Pratt, R. F. (1998) *Biochem. J. 332*, 755−761.
10. Toney, J. H., Hammond, G. G., Leiting, B., Prior, K. D., Wu, J. K., Cuca, G. C., and Pompliano, D. L. (1998) *Anal. Biochem. 255*, 113−119.
11. van Heijenoort, Y., Derrien, M., and van Heijenoort, J. (1978) *FEBS Lett. 89*, 141−144.
12. Suzuki, H., van Heijenoort, Y., Tamura, T., Mizoguchi, J., Hirota, Y., and van Heijenoort, J. (1980) *FEBS Lett. 110*, 245−249.
13. van Heijenoort, Y., Leduc, M., Singer, H., and van Heijenoort, J. (1987) *J. Gen. Microbiol. 133*, 667−674.
14. Ritzeler, O., Hennig, L., Findeison, M., Welzel, P., Müller, D., Markus, A., Lemoine, G., Lampilas, M., and van Heijenoort, J. (1997) *Tetrahedron 53*, 1675−1694.
15. Kurz, M., Guba, W., and Vértesy, L. (1998) *Eur. J. Biochem. 252*, 500−507.
16. Kempin, U., Hennig, L., Welzel, P., Marzian, S., Müller, D., Fehlhaber, H.-W., and Markus, A. (1995) *Tetrahedron 51*, 8471−8482.
17. Adam. M., Fraipont, C., Rhazi, N., Nguyen-Distèche, M., Lakaye, B., Frére, J.-M., Devreese, B., Van Beeumen, J., van Heijenoort, Y., van Heijenoort, J., and Ghuysen, J.- M. (1997) *J. Bacteriol. 179*, 6005−6009.
18. O'Callaghan, C. H., Morris, A., Kirby, S. M., and Shingler, A. H. (1972) *Antimicrob. Agents Chemother. 1*, 283−288.
19. Huber, G., Schacht, U., Weidenmüller, H. L., Schmidt-Thomé, J., Duphorn, J., and Tschesche, R. (1965) *Antimicrob. Agents Chemother.* 737−742.
20. Mast, R. C., and Haynes, L. V. (1975) *J. Colloid Interfac. Sci. 53*, 35−41.
21. De Vendittis, E., Palumbo, G., Parlato, G., and Bocchini, V. (1981) *Anal. Biochem. 115*, 278−286.
22. Bordier, C. (1981) *J. Biol. Chem. 256*, 1604−1607.
23. Wang, C.-C., Schultz, D. E., and Nicholas, R. A. (1996) *Biochem. J. 316*, 149−156.
24. Tanford, C. (1980) in *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, Chapter 7, Wiley-Interscience, New York.
25. Stafford, R. E., Fanni, T., and Dennis, E. A. (1993) *Biochemistry 28*, 5113−5120.
26. Rogers, H. J., Perkins, H. R., and Ward, J. B. (1980) in *Microbial Walls and Membranes*, Chapter 3, Chapman and Hall, London.

27. Volke, F., Waschipky, R., Pample, A., Donnerstag, A., Lantzch, G., Pfeiffer, H., Richter, W., Klose, G., and Welzel, P. (1997) *Chem. Phys. Lipids 85*, 115−123.
28. Berg, O. G., Rogers, R., Yu, B. Z., Yao, J., Romsted, L. S., and Jain, M. R. (1997) *Biochemistry 36*, 14512−14530.
29. Donnerstag, A., Hennig, L., Findeisen, M., Welzel, P., and Haessner, R. (1996) *Magn. Res. Chem. 34*, 1031−1035.
30. Tanford, C. (1980) in *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, Chapter 14, Wiley-Interscience, New York.
31. Ashbrook, J. D., Spector, A. A., Santos, E. C., and Fletcher, J. E. (1975) *J. Biol. Chem. 250*, 2333−2338.
32. Robinson, N. C., and Tanford, C. (1975) *Biochemistry 14*, 369−377.
33. Gow, A., Auton, W., and Smith, R. (1990) *Biochemistry 29*, 1142−1147.
34. von Eijk, J. H., Verheij, H. M., Dijkman, R., and de Haas, G. H. (1983) *Eur. J. Biochem. 132*, 183−188.
35. Hille, J. D. R., Egmond, M. R., Dijkman, R., van Oort, M. G., Jirgensons, B., and de Haas, G. H. (1983) *Biochemistry 22*, 5347−5353.
36. van Oort, M. G., Dijkman, R., Hille, J. D. R., and de Haas, G. H. (1985) *Biochemistry 24*, 7987−7993.
37. van Oort, M. G., Dijkman, R., Hille, J. D. R., and de Haas, G. H. (1985) *Biochemistry 24*, 7993−7999.
38. Mascher, E., and Lundahl, P. (1989) *J. Chromatogr. 476*, 147−158.
39. Yuan, W., Quinn, D. M., Sigler, P. B., and Gelb, M. H. (1990) *Biochemistry 29*, 6082−6094.
40. Steinhardt, J., Scott, J. R., and Birdi, K. S. (1997) *Biochemistry 16*, 718−725.
41. Daban, J.-R., Samsó, M., and Bartolomé, S. (1991) *Anal. Biochem. 199*, 162−168.
42. Richieri, G. V., Ogata, R. T., and Kleinfeld, A. M. (1992) *J. Biol. Chem. 267*, 23495- 23501.
43. Althaus, I. W., Franks, K. M., Langley, K. B., Kézdy, F. J., Peterson, T., Buxser, S. E., Decker, D. E., Dolak, D. A., Ulrich, R. G., and Reusser, F. (1996) *Experientia 52*, 329−335.
44. Osieki-Newman, K., Fabbro, D., Legler, G., Desnick, R. J., and Grabowski, G. (1987) *Biochim. Biophys. Acta 915*, 87−100.
45. Kurihara, H., Tada, S., Takahashi, K., and Hatano, M. (1996) *Biosci. Biotechnol. Biochem. 60*, 932−933.
46. Kilby, P. M., Primrose, W. U., and Roberts, G. C. K. (1995) *Biochem. J. 305*, 935−944.
47. Peters, A. R., Dikker, N., van den Berg, L., Boelens, R., Kaptein, R., Slotboom, A. J., and de Haas, G. H. (1992) *Biochemistry 31*, 10024−10030.
48. van den Berg, B., Tessari, A., Boelens, R., Dijkman, R., de Haas, G. H., Kaptein, R., and Verheij, H. M. (1995) *Nat. Struct. Biol. 2*, 402−406.
49. Davidson, F. F., Hajdu, J., and Dennis, E. A. (1986) *Biochem. Biophys. Res. Commun. 137*, 587−592.
50. Nakagawa, J., Tamaki, S., Tomioka, S., and Mitsuhashi, M. (1984) *J. Biol. Chem. 259*, 13937−13946.
51. Nicholas, R. A., Lamson, D. R., and Schultz, D. E. (1993) *J. Biol. Chem. 268*, 5632−5641.
52. Hammes, W. P., and Neuhaus, F. C. (1974) *Antimicrob. Agents Chemother. 6*, 722−728.
53. Sanderman, H., Jr. (1976) *Biochim. Biophys. Acta 444*, 783−788.
54. Babczinski, P. (1980) *Eur. J. Biochem. 112*, 53−58.
55. Villemez, C. L., and Carlo, P. L. (1980) *J. Biol. Chem. 255*, 8174−8178.
56. Pan, Y. T., and Elbein, A. D. (1996) *Arch. Biochem. Biophys. 335*, 258−266.
57. Hara, H., and Suzuki, H. (1984) *FEBS Lett. 168*, 155−160.
58. DiBerardino, M., Dijkstra, A., Stüber, D., Keck, W., and Grubler, M. (1996) *FEBS Lett.* 184−188.
59. Marzian, S., Happel, M., Wagner, U., Müller, D., Welzel, P., Fehlhaber, H.-W., Stark, A., Schütz, H.-J., Markus, A., and Limbert, M. (1994) *Tetrahedron 50*, 5299−5308.

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