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Activation for Catalysis of Penicillin-Binding Protein 2a from Methicillin-Resistant *Staphylococcus aureus* by Bacterial Cell Wall

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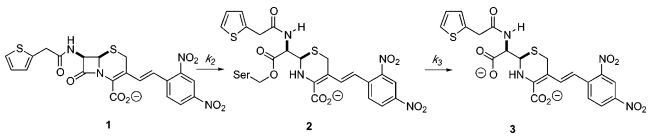
Methicillin-resistant *Staphylococcus aureus* ("MRSA") is a leading cause of bacterial infections and a global scourge.[1,2] The recently emerged vancomycin-resistant variants of MRSA are among the most challenging bacterial strains for chemotherapeutic intervention.[3−6] MRSA strains are resistant to all available β-lactam antibiotics by the virtue of their acquisition of the *mec*A gene, which encodes penicillin-binding protein 2a (PBP 2a).[2,7]

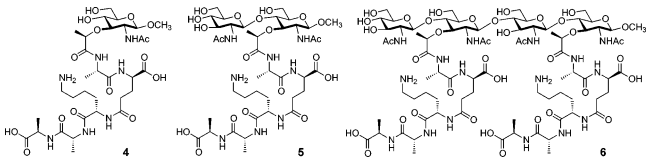
β-Lactam antibiotics acylate a critical active-site serine in PBPs. This reaction is depicted below for nitrocefin (**1**), a chromogenic cephalosporin (a β-lactam; **1** → **2**) used in our study. The acyl-enzyme species (**2**) undergoes very slow hydrolysis (to give **3**); hence, its longevity deprives bacteria of the critical functions of PBPs leading to the bactericidal activities of β-lactam antibiotics. The *S. aureus* PBP 2a is refractory to the action of available β-lactam antibiotics since it has a closed active site, according to the X-ray structure.[8] The lack of access to the active-site serine manifests itself in high dissociation constants (K_s) for the preacylation complex and small rate constants for acylation (k_2).[7] However, PBP 2a continues to catalyze its physiological reaction, the cross-linking of the *S. aureus* cell wall, unencumbered.[1,2] In essence, PBP 2a resists modification by β-lactam antibiotics, but remains a competent catalyst for its physiological reaction. The advantage of such an enzyme for bacterial survival in the face of the challenge by these antibiotics is self-evident, but how PBP 2a possesses such differential effects toward its substrate and inhibitors is presently not understood.



PBPs utilize the polymeric bacterial cell wall, also known as the peptidoglycan, as substrate. We reasoned that surface interactions of the polymeric substrate outside the closed active site must facilitate the opening of the active site to make the entry of the peptidoglycan possible. This interaction would be absent with the small-molecule β-lactam antibiotics. Availability of three analogues of the bacterial peptidoglycan fragments (compounds **4**−**6**) in our lab, each prepared in multistep syntheses,[9,10] provided an opportunity to explore this possibility. Compound **6** has four residues of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). The NAM residues have been appended with the pentapeptide (NAM-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala) seen in many bacteria. In some *S. aureus*, the γ-D-Glu, is substituted with γ-D-Gln (we have prepared derivatives with this change in structure, compounds **7** and **8**, which behave the same in interactions with PBP 2a; Supporting Information). The peptidoglycan of *S. aureus* has a pentaglycyl moiety, the site of the cross-linking reaction of PBP 2a, appended to the side chain of L-Lys. We have deliberately left this moiety out of the structures, as we did not want the compounds to be turned over by PBP 2a. The synthetic compounds **4** and **5** are smaller fragments of compound **6**.

Table 1. Kinetic Parameters for Interactions of Nitrocefin (**1**) with PBP 2a in the Absence and Presence of Compounds **4**, **5**, and **6**

compound (mM)		k_2 (s^{-1}) × 10^3	K_s (μM)	k_2/K_s ($M^{-1}s^{-1}$)	k_3 (s^{-1}) × 10^6
	0	3.7 ± 0.3	192 ± 24	19 ± 3	7.2 ± 0.1
4	0.5	15 ± 1	160 ± 30	90 ± 20	35 ± 2
	1.0	25 ± 5	110 ± 30	180 ± 50	
	1.5	30 ± 5	95 ± 10	295 ± 60	
	2.0	30 ± 1	80 ± 10	380 ± 45	
	3.0	35 ± 5	45 ± 10	700 ± 130	100 ± 4
5	0.5	25 ± 5	185 ± 30	140 ± 20	28 ± 1
	1.0	40 ± 15	160 ± 30	250 ± 55	
	1.5	55 ± 15	120 ± 30	460 ± 150	
	2.0	65 ± 15	100 ± 15	650 ± 130	
	3.0	80 ± 20	75 ± 10	1100 ± 200	80 ± 2
6	0.5	25 ± 10	175 ± 60	130 ± 45	30 ± 2
	1.0	35 ± 5	140 ± 30	260 ± 55	
	1.5	60 ± 10	115 ± 5	440 ± 90	
	2.0	85 ± 10	95 ± 10	900 ± 85	60 ± 8



We have used nitrocefin (**1**), a chromogenic β-lactam, as a reporter molecule to evaluate the effects of the peptidoglycan fragments on PBP 2a. The methodology for cloning, expression, and purification to homogeneity of PBP 2a and determination of k_2, k_3, and K_s with β-lactam antibiotics have been reported.[7] Table 1 (first line) gives the kinetic parameters for interactions of nitrocefin with PBP 2a. Enzyme acylation (k_2) proceeds slowly ($t_{1/2}$ of 3.1 min) and deacylation (k_3) is exceedingly slow ($t_{1/2}$ of 26.7 h). In the presence of the peptidoglycan fragments **4**−**6** certain trends were established. As the concentration of a given peptidoglycan fragment was increased, the rate constant for acylation (k_2) of PBP 2a by nitrocefin was enhanced, and the value of the dissociation constant (K_s) was attenuated in each case. These observations indicated that in the presence of increasing concentrations of the peptidoglycan fragments, the active site became more available to the β-lactam antibiotic, as judged by both the enhanced facility of enzyme acylation and by improvement of the stability of the noncovalent preacylation complex of the antibiotic and PBP 2a. The rate constants for deacylation (k_3) were also enhanced by approximately 3-fold in the presence of the peptidogylcan fragments.

These trends on kinetic parameters argue for a change in protein conformation from that seen in the X-ray structure for the closed

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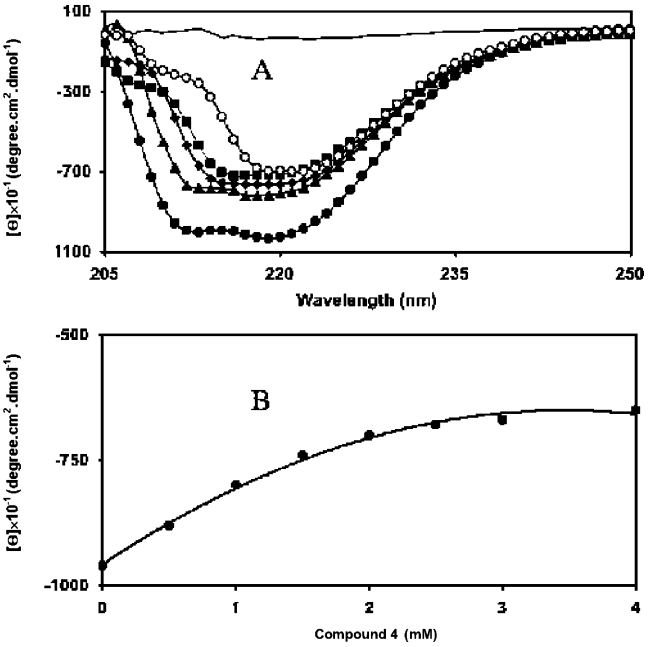

Figure 1. (A) Far-UV circular dichroic spectra of compound **4** by itself (4.0 mM, —), of PBP 2a by itself (3 μM, ●), and of PBP 2a (3 μM) in the presence of **4** at 1.0 mM (▲), 2.0 mM (◆), 3.0 mM (■), and 4.0 mM (○). The lines connect the data points and were not fitted to any specific model. (B) Change in molar ellipticity of PBP 2a at 222 nm as a function of the concentrations of compound **4**.

active site to one that makes the active site more accessible to nitrocefin in the presence of the peptidoglycan fragments. We reasoned that a change in protein conformation should be detectable by circular dichroic (CD) measurements. As shown in Figure 1, compound **4** does not have any far-UV chromophore in the range of wavelengths for the study of the protein. The CD spectrum of PBP 2a (Figure 1A) shows two strong minima at 208 and 222 nm corresponding to α-helices typically seen in this family of proteins. On addition of compound **4** to the solution of PBP 2a, the protein undergoes a conformation change consistent with a decrease in the degree of helicity. This process is saturable (Figure 1B) with respect to the analogue of the cell wall fragment. The change in the conformation is likely not limited to mere decrease in helicity, but a more definitive analysis of the structural consequences of binding of the peptidoglycan fragments to PBP 2a should await X-ray analyses of these complexes. The results of the CD studies with compounds **5** and **6** paralleled those with **4** (see Supporting Information).

Double reciprocal plots of the concentrations of peptidoglycan fragments versus k_2 values furnished the dissociation constants for the peptidoglycan fragments (K_d). The K_d values for compounds **4**, **5**, and **6** were 1.0 ± 0.4, 2.0 ± 0.8, and 2.8 ± 1.0 mM, respectively. In light of the saturation seen in the CD experiments (Figure 1B), a similar analysis was carried out by CD for compounds **4** and **5** (the available quantity of **6**, which was synthesized in 37 steps, was not sufficient for this analysis). This analysis furnished K_d values of 1.1 ± 0.2 and 1.4 ± 0.2 mM for compounds **4** and **5**, respectively. The two independent methods furnish similar dissociation constants for the noncovalent complex of the peptidoglycan fragments and PBP 2a.

These K_d values for the peptidoglycan fragments are in the millimolar range. We have argued previously that because of favorable entropic factors in interactions of cell wall with many PBPs, the effective concentrations of the cell wall components that the PBPs experience on the bacterial cytoplasmic membrane are high.[9,11] As such, there might not have been compelling reasons for evolution of enhanced affinity for the cell wall by many of these enzymes. This is true for many important physiological processes. Regardless, it is likely that the K_d value for a much larger fragment of peptidoglycan, one that would approach a polymeric character or one with the pentaglycyl moiety appended to the L-Lys residue, would be lower than those measured for compounds **4**−**6**. This would make the process more efficient, such that the many cross-linking events of the cell wall could proceed unencumbered within the 20−30 min required for the doubling of the population of *S. aureus* growing under favorable conditions.

What has been presented above describes a model for activation of PBP 2a for its catalytic function consistent with interactions of the cell wall, its polymeric substrate, outside the active site. Lee et al. have presented X-ray structural evidence for another PBP that indicates that for the cross-linking reaction, the enzyme is capable of sequestering sequentially two portions of the cell wall in grooves on the surface of the enzyme where, at the closest point, the two grooves are within 20 Å of each other.[12] We believe that similar binding sites for the peptidoglycan exist for PBP 2a. The binding of the first peptidoglycan piece to the surface outside the active site would appear to stimulate the opening of the active site to allow catalysis to commence.

It has not escaped us that such stimulatory activity of the peptidoglycan on PBP 2a may be exploited in sensitizing the protein toward inhibition by the existing β-lactam antibiotics. Such a strategy would entail identifying a suitable mixture of a peptidoglycan fragment and a β-lactam antibiotic that would work in concert in reversing the deleterious β-lactam-resistant phenotype of MRSA.

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Supporting Information Available: Experimental procedures, including those for kinetics and synthetic protocols and characterization of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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