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A Novel β-Lactamase Activity from a Penicillin-binding Protein of *Treponema pallidum* and Why Syphilis Is Still Treatable with Penicillin*

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***Treponema pallidum*, the causative agent of syphilis, is sensitive to penicillins. Yet, an abundant membrane-bound protein of this organism, Tp47, turns over penicillins. It is shown herein that the turnover process is a hydrolytic reaction that results in the corresponding penicilloates, products that have their β-lactam bonds hydrolyzed. This is the reaction of β-lactamases, *bona fide* resistance enzymes to β-lactam antibiotics. Remarkably, the x-ray structure of Tp47 bears no resemblance to any other β-lactamases or the related penicillin-binding proteins. Furthermore, evidence is presented that the reaction of Tp47 takes place in the absence of the zinc ion and does not involve intermediary acyl enzyme species. Hence, the β-lactamase activity of Tp47 is the fifth known mechanism for turnover of β-lactam antibiotics. Tp47 also exhibits a penicillin binding reaction, in the process of which the enzyme is covalently modified in the active site. The two reactions take place in two different active sites, and the events of the β-lactamase activity are over 2,000-fold more rapid than the penicillin binding reaction. The level of β-lactamase activity is high and is held back only by a strong product-inhibition component to the catalytic process. If natural selection would result in a mutant variant of Tp47 that overcomes product inhibition for the β-lactamase activity, a novel *bona fide* resistance to penicillins will emerge in *Treponema*, which will be a disconcerting clinical development. The physiological functions of Tp47 are not known, but it is likely that this is at least a bifunctional enzyme involved in the processing of the *Treponema* peptidoglycan as a substrate.**

Treponema pallidum is the causative agent of syphilis, a chronic debilitating disease that has been known for centuries. Syphilis is exquisitely sensitive to penicillins and other β-lactam antibiotics, but the lethal targets of these drugs are not known in this organism. Incubation of membrane fractions with radioactive penicillin has identified several penicillin-binding proteins (1) for which the biochemical analyses have not been carried out. Furthermore, no β-lactamase activity, the primary cause of resistance to β-lactam antibiotics, has been identified for this organism or predicted from the genomic information on *T. pallidum*.

A novel 47-kDa membrane-bound lipoprotein from this organism, named Tp47, has been shown to experience covalent modification by penicillin; hence it has been designated a penicillin-binding protein (2, 3). It is the most abundant membrane protein in *T. pallidum*, and it has no sequence homology to any other bacterial or eukaryotic protein (2). Furthermore, its recently elucidated x-ray structure reveals it to be distinct from any known penicillin-binding protein or from the related β-lactamases (2). This protein has four distinct domains, and its active site(s) has not been identified.

An earlier investigation described the ability of this protein to turn over β-lactam antibiotics (2). We have explored this initial observation further in this study. By analyzing the products of this turnover chemistry, we report herein that Tp47 hydrolyzes the β-lactam bonds of penicillins. Furthermore, we disclose that the reaction is unique and represents a fifth mechanistic strategy in the turnover of these antibiotics. We note that the activity of Tp47 as a β-lactamase is held back by a significant inhibition of the activity by the products of turnover, accounting for the sensitivity of *T. pallidum* to penicillins. Furthermore, we have evaluated the kinetics of the penicillin binding reaction that leads to covalent modification of the protein. The penicillin binding and the β-lactamase reactions take place at different active sites in this protein, with the latter reaction proceeding at a rate of over 2,000-fold faster than that of the former.

EXPERIMENTAL PROCEDURES

Investigation of the Reaction of Tp47 with a Penicillin and Purification of the Reaction Product—Recombinant Tp47, a gift from the laboratory of Michael Norgard, was purified as described earlier (2). A total of 5 mg of Tp47 was incubated with 3 mg of oxacillin for 5 h at 25 °C. The reaction mixture was passed through a centricon device (Ultrafree Biomax, 5 kDa, Millipore) to remove the protein from the solution. The solution was put through HPLC[1] for purification of the product of turnover (C_{18} column, Waters, Deltapak 15 μm, 300 × 19 mm for purification, linear gradient 0–96% methanol in water, and 0.1% trifluoroacetic acid solution over 45 min, 5 ml/min). The reaction product (oxacillin turnover product t_R 22.8 min) and oxacillin (t_R 37.1 min) were separable. The samples from HPLC were collected, and the solvent was evaporated under high vacuum. ^{1}H NMR (500 MHz, CD_3OD) δ 1.31 (s, 3H), 1.60 (s, 3H), 2.69 (s, 3H), 3.60 (s, 1H), 4.54 (d, 1H, J = 5.0 Hz), 5.20 (d, 1H, J = 5.0 Hz), 7.48–7.49 (m, 3H), 7.86–7.87 (m, 2H); MS ESI$^+$ 420 (M − 2Na + H).

Once it was clear that the reaction of Tp47 with oxacillin was hydrolysis of the β-lactam bond, we used the TEM-1 β-lactamase from *Escherichia coli* to turn over larger quantities of two penicillins. The TEM-1 β-lactamase (2–3 mg) was incubated with 30 mg of oxacillin or carbenicillin under the same condition described for Tp47, and the HPLC conditions were the same (linear gradient 0–96% methanol in water and 0.1% trifluoroacetic acid solution over 45 min, 5 ml/min). The retention times for oxacillin and its hydrolytic product are given above. Retention times for carbenicillin hydrolytic product and carbenicillin itself are t_R 16.0 min and t_R 33.5 min, respectively. The characteristics

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[1] The abbreviation used is: HPLC, high pressure liquid chromatography.

for the turnover product of carbenicillin are as follows: ^{1}H-NMR (500 MHz, CD_3OD) δ 1.29 (s, 3H), 1.52 (s, 3H), 3.64 (s, 1H), 4.33 (s, 1H), 4.37 (d, 1H, J = 2.4 Hz), 5.25 (d, 1H, J = 3.95 Hz), 7.32 (m, 3H), 7.62 (m, 2H); MS ESI$^+$ 397 (M − 3Na + H).

Attempted Stopped-flow Kinetics—The experiments were carried out on a Cary 50 UV spectrophotometer (Varian Inc.) equipped with an SFA-20 stopped-flow apparatus (Hi-Tech Scientific, Salisbury, UK) at room temperature. Attempts were made at stopped-flow kinetics with ampicillin, penicillin G, carbenicillin, oxacillin, and cephalosporin C. Each substrate and the purified Tp47 were prepared in Buffer A (20 mM Hepes, 20 mM NaCl, pH 7.4). We tried to observe the progress of potential acylation at 235 nm in the case of ampicillin, penicillin G, and carbenicillin and at 260 nm with oxacillin and cephalosporin C. Multiple trials were made with several ratios of enzyme and substrate. The initial trial was with 2–50 μM substrate and 1 μM Tp47. Others were performed with 2–10 μM substrate with 100 nM Tp47. All reactions were monitored from 20 ms to 10–15 min.

Steady-state Kinetics—The steady-state kinetics of the β-lactamase activity of Tp47 was carried out with several β-lactam antibiotics. The protein was incubated with the β-lactam in Buffer A, and the catalytic activity was measured on a Cary 50 (Varian Inc.) UV-visible spectrophotometer. The molar extinction coefficients for turnover of substrates are as follows: penicillin G, $\Delta\epsilon_{240} = -560\ M^{-1}\,cm^{-1}$; ampicillin, $\Delta\epsilon_{235} = -820\ M^{-1}\,cm^{-1}$; carbenicillin, $\Delta\epsilon_{235} = -780\ M^{-1}\,cm^{-1}$; oxacillin, $\Delta\epsilon_{260} = +258\ M^{-1}\,cm^{-1}$; cloxacillin, $\Delta\epsilon_{260} = +140\ M^{-1}\,cm^{-1}$; BOCILLIN FL, $\Delta\epsilon_{504} = +490\ M^{-1}\,cm^{-1}$; **1**, $\Delta\epsilon_{235} = -430\ M^{-1}\,cm^{-1}$; **2**, $\Delta\epsilon_{235} = -430\ M^{-1}\,cm^{-1}$; **3**, $\Delta\epsilon_{235} = -420\ M^{-1}\,cm^{-1}$; **4**, $\Delta\epsilon_{235} = -420\ M^{-1}\,cm^{-1}$; **5**, $\Delta\epsilon_{235} = -510\ M^{-1}\,cm^{-1}$; **6**, $\Delta\epsilon_{235} = -510\ M^{-1}\,cm^{-1}$. The turnover rates were calculated from the initial 5–10% from the spectrum of the reactions at various concentrations of substrates. The K_m and k_{cat} values were determined by fitting the data according to the methods of Lineweaver-Burk and Hanes-Woolf. Both methods produced similar values for the parameters.

Inhibition Kinetics with Purified Products—Two concentrations of ampicillin (20 and 50 μM) in Buffer A were used for the study of inhibition of Tp47 by the hydrolytic turnover products of oxacillin and carbenicillin. The concentrations of the turnover products were 0–60 μM for each assay. Tp47 (20 nM) was incubated with the reaction product for 5 min prior to the addition of ampicillin and the monitoring of its hydrolysis at 235 nm. The inhibitor constants were determined by the method of Dixon (4).

Demonstration of Full Activity after the β-Lactamase Reaction—A reaction of 2 μM Tp47 and 50 μM carbenicillin was set up in Buffer A and was allowed to reach full inhibition of the β-lactamase activity (7–15 min). A portion (5 μl) of the reaction mixture was diluted 100-fold into Buffer A containing 20 μM carbenicillin, and the progress of the reaction was monitored by UV spectrophotometer at 235 nm. Another portion (30 μl) of the incubation mixture was passed through a desalting column (Micro Bio-Spin®6 column, Bio-Rad) to remove the β-lactam. Subsequently the solution was diluted 100-fold into Buffer A with 20 μM carbenicillin, and the turnover progress was monitored by spectrophotometer at 235 nm. The two hydrolysis profiles turned out to be very similar (see "Results and Discussion").

Penicillin Binding Assay—This binding assay was tested with fluorescent penicillin BOCILLIN FL (5). Several reaction mixtures were set up in Eppendorf tubes, each containing 2 μM Tp47 and BOCILLIN FL (50, 100, 200, or 250 μM) in a total volume of 20 μl. Each tube was incubated for 0, 5, 10, 15, 20, and 60 min at 37 °C, at which point the reactions were quenched by the addition of 15 μl of the SDS sample buffer (125 mM Tris, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, pH 6.8), and the mixtures were boiled for 4 min. The samples were loaded onto 15% SDS-PAGE, and the gel was developed and scanned using a Storm 840® Fluorimager. The fluorescent bands were analyzed by ImageQuant 5.2 software. The observed first-order rate constants (k_{obs}) for acylation of Tp47 by BOCILLIN FL were determined by fitting the change in intensity of the fluorescent bands as a function of time to the following equation: $A_t = A_{max}(1 - \exp(-k_{obs}t))$, where A_t is the intensity at time t. The k_{obs} values were plotted against BOCILLIN FL concentrations to determine the first-order rate constant (k_2) for acylation and the dissociation constant K_d with the following equation: $k_{obs} = k_2 \times [S]/(K_d + [S])$. The following equation describes the penicillin binding reaction of the Tp47, where ES is the noncovalent preacylation complex, E-S is the acyl enzyme species, and S and P stand for substrate and product.

$$E + S \underset{}{\overset{K_d}{\rightleftharpoons}} E\text{S} \overset{k_2}{\longrightarrow} E\text{-S} \overset{k_3}{\longrightarrow} E + \text{P} \qquad \text{(Eq. 1)}$$

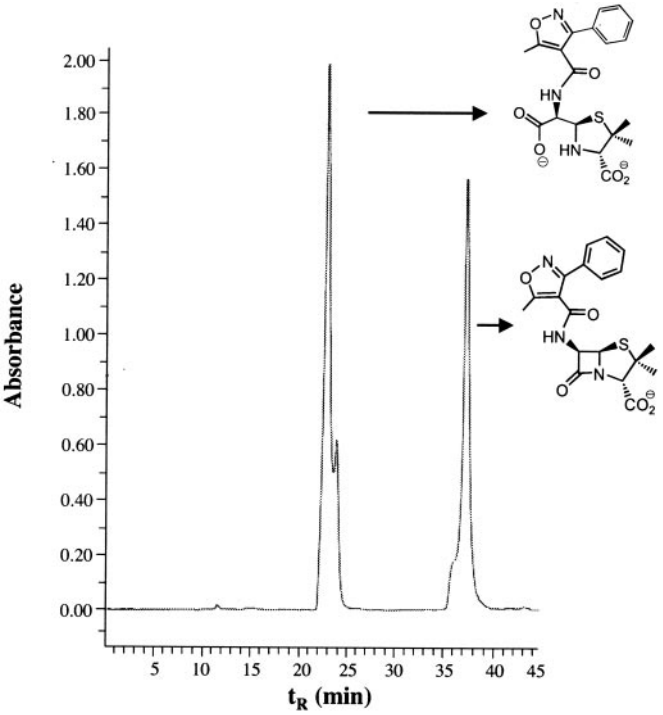


FIG. 1. **HPLC analysis of the turnover of oxacillin by Tp47.** For HPLC conditions, see "Experimental Procedures."

RESULTS AND DISCUSSION

The initial report of the β-lactamase activity from *T. pallidum* was intriguing to us for a number of reasons. First, the structure of the enzyme bore no resemblance to any known β-lactamase of any class, of which there are four. Second, the sensitivity of the organism to penicillin in the face of this activity was paradoxical. We note that the earlier report was based on the observation of spectrophotometric changes in solutions of the β-lactam antibiotics in the presence of Tp47, and a direct hydrolysis of the β-lactam nucleus was not documented. As such, it was necessary that we demonstrate that a *bona fide* β-lactamase reaction was in hand.

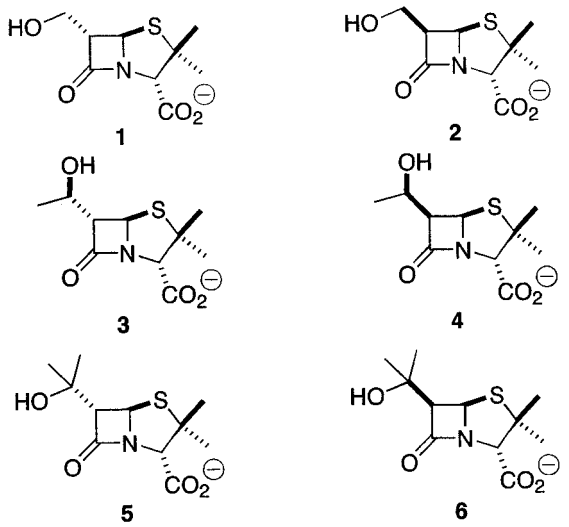
As described under "Experimental Procedures," we incubated oxacillin, a penicillin, with Tp47. We were able to document a clear conversion of the antibiotic to a new species. The new compound was purified by HPLC, and our structural characterization by NMR and mass spectrometry revealed that indeed the reaction of Tp47 with this antibiotic was a β-lactamase reaction (Fig. 1). Hence, a *bona fide* β-lactamase reaction with Tp47 has now been documented.

There are four classes of known β-lactamases described in the literature (6–12). Classes A, C, and D are active-site serine enzymes. These enzymes experience acylation of their active-site serine by the β-lactam antibiotic, which then undergoes deacylation in the second step of the reaction. Each class has developed a distinct mechanism for deacylation, but they share the acylation step, which has been handed down in an evolutionary sense from the parental PBP (6–9).

Class B β-lactamases are zinc ion-dependent and undergo their reactions by a distinct chemistry that does not involve covalent chemistry in the course of the turnover reaction (13, 14). The structure of Tp47 bears no resemblance to those of the class B β-lactamases. Furthermore, Deka *et al.* (2) have observed that the x-ray structure of Tp47 does not contain a zinc ion or any other transition metal ion. It is conceivable that Tp47 may bind a metal ion, such as the zinc ion, but that it was not seen in the crystallographic analysis. We carried out the β-lactamase reaction after the enzyme was incubated with

TABLE I
Kinetic parameters for turnover of β-lactam antibiotics by Tp47
The assay was performed in 20 mM Hepes, 20 mM NaCl, pH 7.4. The concentration of protein in the assays was 20 nM. (See "Experimental Procedures" for more details.)

Substrate	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	$(M^{-1}\ s^{-1}) \times 10^{-5}$
Penicillin G	5.9 ± 0.3	36.4 ± 2.5	1.6 ± 0.1
Ampicillin	6.8 ± 1.2	12.1 ± 3.4	5.6 ± 1.8
Carbenicillin	11.7 ± 2.6	19.5 ± 3.6	6.0 ± 1.6
Oxacillin	3.7 ± 0.6	95.5 ± 15.6	0.4 ± 0.1
Cloxacillin	5.0 ± 0.6	96.7 ± 12.8	0.5 ± 0.1
BOCILLIN FL	5.0 ± 0.2	6.9 ± 0.7	7.2 ± 0.7
1	11.5 ± 1.0	1.3 ± 0.3	89 ± 20
2	10.6 ± 0.9	2.5 ± 0.6	42 ± 10
3	11.5 ± 0.6	3.3 ± 0.6	35 ± 6
4	10.8 ± 2.1	3.9 ± 1.4	28 ± 11
5	8.0 ± 1.8	20.8 ± 7.2	3.8 ± 1.6
6	17.0 ± 1.7	28.6 ± 4.9	5.9 ± 1.1



SCHEME 1

EGTA, a metal ion chelator, and saw no effect on the rate of the reaction. Hence, it would appear that there is no contribution by a zinc ion to the turnover chemistry, and the β-lactamase reaction of enzyme is by necessity distinct from that of class B β-lactamases. We reached this conclusion, because of our demonstration of the β-lactamase reaction above without supplementing the medium with the zinc ion, and the protein itself is devoid of it, as per crystal structure.

We attempted stopped-flow kinetics to identify transient accumulation of any potential acyl enzyme species with Tp47 under conditions of single turnover chemistry within the time frame of 20 ms to several minutes. These efforts all failed to identify such a species. Whereas one might argue that the transient formation and the subsequent hydrolysis of the potential acyl enzyme species could have taken place prior to the first kinetic measurement at 20 ms (the limit of our instrument), such a set of events in that time frame would be inconsistent with the measured steady-state kinetic parameters (discussed below).

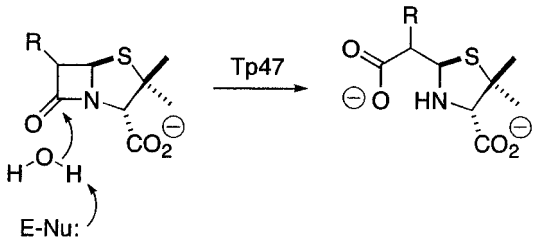
We carried out steady-state analyses for turnover of β-lactam antibiotics (Table I). With initial rate measurements, we were able to evaluate respectable levels of activity with penicillins. The k_{cat}/K_m values were in the range of 10^5–10^6 M^{-1} s^{-1}, which should be sufficient for the manifestation of the phenotypic observation of resistance for a protein as abundant as Tp47. It is noteworthy that the K_m values are in the micromolar range, which are attainable *in vivo*, so saturation should not be a problem for Tp47 when a *T. pallidum* infection is being treated with penicillin. It was interesting that we were not able to measure any activity against cephalosporins. Furthermore, we could not even detect inhibition of the turnover of penicillins in the presence of three cephalosporins, cephalosporin C, cephalothin, and cephaloridine. Therefore, the Tp47 β-lactamase activity would appear to be limited to penicillins.

We have reported several uses of a novel set of synthetic hydroxyalkylpenicillanate derivatives that have been proven versatile in arresting catalysis at the acyl enzyme stage of the β-lactamase reaction (15–21). In essence, the presence of the hydroxyalkyl moiety at position 6 of the penicillin nucleus would prevent the travel of the hydrolytic water toward the ester moiety of the acyl enzyme species, and hence the second step of catalysis would be impaired. Furthermore, the larger the hydroxyalkyl group, the more dramatic the effect, as the hindrance to the travel of the hydrolytic water is more severe. These compounds were expressly designed to prevent hydrolysis at the stage of the acyl enzyme species. If the water molecule approaches the acyl enzyme species from the α direction (the case of β-lactamases of classes A and D), then a 6α-hydroxyalkylpenicillanate (compounds **1**, **3**, and **5**) would be useful. Conversely, a 6β-hydroxyalkylpenicillanate (compounds **2**, **4**, and **6**) would interfere with the travel of the hydrolytic water molecule from the β direction (the case of class C β-lactamases). We have used this type of compound previously in numerous mechanistic and crystallographic studies of the acyl enzyme species in β-lactamases (15–21).

We had hoped that the use of both the α- and β-type of these hydroxyalkylpenicillanates would have not only trapped a potential acyl enzyme species in the course of catalysis by Tp47 but also would have guided us in elucidating the direction of the approach of the hydrolytic water molecule to the ester moiety of the acyl enzyme species. Not only did compounds **1**–**6** not give rise to stable acyl enzyme species, they all served as effective substrates for Tp47 (Table I). It is remarkable that the kinetic parameters for any of the three pairs of the synthetic penicillanate derivatives are essentially the same within the experimental limits of standard deviations. This clearly indicates that the presence of the hydroxyalkyl group on the penicillanate nucleus, whether on the α or β side, makes no difference. We hasten to add that we would have expected that the hydroxyalkyl group of one of the stereoisomers in each pair would serve its function in prevention of deacylation, whereas the other should have served as a substrate. The fact that both molecules in each pair served as equally good substrates for Tp47 underscores the absence of an acyl enzyme species in the enzymic reaction (Scheme 1).

Collectively, the lack of observation of any accumulating species during stopped-flow experiments and the results with turnover of compounds **1**–**6** all argue that acyl enzyme species do not exist in the course of the turnover chemistry of β-lactam antibiotics by Tp47. Hence, we concluded that the β-lactamase reaction of Tp47 does not go through an intermediary acyl enzyme species, nor is it dependent on a zinc ion. Furthermore, because the x-ray structure for the protein is also unique, we concluded that this β-lactamase activity is a distinct activity for turnover of these antibiotics without any precedent.

The turnover data clearly indicate the presence of saturation in the turnover chemistry. Therefore, penicillins are binding to a specific binding site, that is, an active site, on the surface of the enzyme in the process of their turnover. The physiological role for Tp47 has not been elucidated, but it is likely that it is a penicillin-binding protein involved in interactions with the peptidoglycan components of the *Treponema* cell wall (3, 22). Tipper and Strominger (23) argued a number of years ago that the acyl-D-Ala-D-Ala portion of the peptidoglycan structure is



SCHEME 2

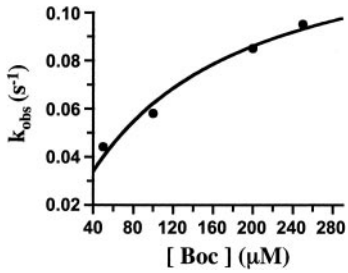


FIG. 2. **The observed first-order rate constants for covalent modification of Tp47 as a function of the concentrations of BOCILLIN FL.** The equation that defines this plot is described under "Experimental Procedures."

nicely mimicked by the backbone of penicillins. Therefore, the chances are that penicillins exploit these molecular recognition events within the active site of Tp47 in binding to the protein. The fact that penicillin undergoes hydrolysis is likely to be an adventitious reaction. Tp47 may be a DD-carboxypeptidase, as suggested earlier (3), and may have the catalytic machinery for activation of the hydrolytic water molecule within the active site. Binding of penicillin within the same site subverts the process by allowing the approach of the activated water to the β-lactam carbonyl to result in hydrolysis of the substrate. However, based on the preceding discussion and evidence, it would appear that the active site-bound penicillin undergoes hydrolysis resulting in the product of the reaction without the involvement of any intermediary steps (Scheme 2).

Now the pertinent question is why, in the face of this β-lactamase activity, *T. pallidum* has remained susceptible to penicillin as a treatment option. The typical change in absorbance for the chromophore in the substrate that is monitored in the turnover process should be a first-order hyperbola. This is the case for turnover of penicillins by Tp47, but we noted that the termination of the reaction was reached in every case well before the anticipated end point based on the change in the extinction coefficient upon hydrolysis of the substrate. In essence, the full hydrolysis of the β-lactam substrate by Tp47 would not take place. One possible explanation for this observation is that a species in the course of the turnover event actually inhibits the enzyme. The proposed mechanism above for turnover of penicillins by Tp47 stipulated an activation of water to give hydrolysis without invocation of any intermediates. Therefore, we chose to study the product itself as the inhibitor of the enzyme.

We turned over relatively large portions of oxacillin and carbenicillin by the TEM-1 β-lactamase, which hydrolyzes them well, and the products of turnover were purified by HPLC. With the availability of the turnover products, we investigated them as inhibitors of the turnover of ampicillin by Tp47. Both products of the turnover of oxacillin and carbenicillin showed competitive inhibition of hydrolysis of ampicillin with dissociation constants (K_i) of 5.9 ± 3.1 μM and 4.0 ± 2.8 μM, respectively. Hence, Tp47 is prone to product inhibition at low micromolar levels of the product. Indeed, insofar as K_m may approximate a dissociation constant, it would appear that the products of the turnover of oxacillin and carbenicillin bind Tp47 better than the antibiotics themselves. Furthermore, the hyperbolic progress curve is due to both the first-order exhaustion of the substrate and progressive inhibition of the enzyme as the product is being formed in each case. This is the reason that initial rate measurements were carried out with these substrates during the steady-state treatment of the turnover process.

Product inhibition for the β-lactamase reaction is accomplished by the formation of a noncovalent complex, as evidenced by the following experiment. This is a point of distinction to the penicillin binding reaction, which will be discussed later. We monitored the profile of the turnover of carbenicillin by Tp47 initially. Another portion of the inhibited protein was dialyzed rapidly to remove the unreacted β-lactam and the product from the solution and from the complex. By so doing, we regenerated the protein, which could turn over carbenicillin again with a similar profile seen initially (data not shown). Therefore, the inhibited complex of the enzyme and the product does not involve a covalent bond.

As mentioned earlier, Tp47 also exhibits a penicillin binding reaction, which involves the formation of a covalent bond between the protein and penicillin, and is a species that enjoys chemical stability. We carried out experiments to evaluate this reaction quantitatively. We used BOCILLIN FL for this experiment, which is a fluorescent penicillin. Typically, the enzyme is incubated with BOCILLIN FL, the protein is subsequently denatured, and the degree of protein modification by the penicillin can be quantified by fluorescence readings as a function of the concentration of the penicillin and of time. These analyses revealed that the penicillin binding reaction takes place with a first-order microscopic rate constant (k_2) of 0.0023 ± 0.0003 s^{-1} and a dissociation constant (K_d) of 124 ± 32 μM (Fig. 2). The K_d value is for the formation of the pre-acylation complex (see "Experimental Procedures"). From the k_2 value, we can determine the $t_{½}$ for the formation of the acylated species at ~300 s.

We also evaluated the kinetic parameters for turnover of BOCILLIN FL by the β-lactamase reaction (Table I), which were k_{cat} = 5.0 ± 0.2 s^{-1} and K_m = 6.9 ± 0.7 μM. The $t_{½}$ for this reaction is ~140 ms. The difference between the turnover rate for the β-lactamase reaction and the rate constant for the formation of the acylated species in the penicillin binding reaction (k_{cat}/k_2), both evaluated with BOCILLIN FL, is 2,170-fold.

It is important to note that, because of this large difference between the rate constants for the two reactions, the two processes do not compete with each other, an assertion that is even more to the point in light of the product inhibition of the β-lactamase reaction. On exposure of Tp47 to penicillin, the enzyme turns it over until the onset of the inhibition of the β-lactamase reaction caused by product inhibition. Only at that point is there an opportunity for the penicillin binding reaction to proceed, an event that takes place substantially more slowly than the β-lactamase activity.

Collectively, these findings argue for the existence of two independent active sites for the two reactions on the surface of Tp47. The K_d for the penicillin binding reaction of BOCILLIN FL is 124 ± 32 μM. Insofar as K_m may approximate a dissociation constant, the corresponding number for the β-lactamase reaction with BOCILLIN FL is 6.9 ± 0.7 μM. Therefore, Tp47 is, at the minimum, a bifunctional penicillin-binding protein with two active sites that exhibit disparate affinities for the same penicillin, and their rate constants for the respective reactions are substantially different from one another. Presumably, the physiological function(s) of this enzyme is the proc-

essing of the bacterial peptidoglycan. Both active sites recognize penicillins in light of the fact that penicillins are structural mimics of the acyl-D-Ala-D-Ala portion of the peptidoglycan. The two physiological functions are yet to be elucidated.

As a concluding note, our observations show that the β-lactamase activity of Tp47 is significant (k_{cat}/K_m values in the range of 10^5 to 10^6 M^{-1} s^{-1}), and it is essentially an activity that has been suppressed by the onset of product inhibition in the course of the turnover of penicillins. This is a dormant activity that has the potential of being unleashed as a consequence of selection of mutant variants of Tp47 that might abolish product inhibition. In the event of the emergence of such mutant variants of Tp47, there will be an innate resistance to penicillins in *T. pallidum*, an event that bodes poorly for the prospects of treatment of syphilis with penicillins.

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