Supporting Information

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SI Materials and Methods

Stability of Acyl-Enzyme over Time. In a 200 μL reaction volume, 1 μM TEM-1 was incubated with or without 5 μM avibactam for 5 min at 37 °C and subjected to two ultrafiltration steps to remove excess inhibitor (Ultrafree-0.5 with Biomax membrane, 5-kDa cutoff; Millipore). Centrifugation at 10,600 × *g* for 8 min was performed at 4 °C. After each ultrafiltration step, 20 μL retentate were diluted with 180 μL assay buffer to restore the original enzyme concentration. TEM-1 activity at each time point was assayed by diluting 1 μM enzyme retentate 4,000-fold in the assay buffer and adding 20 μL 4,000-fold diluted enzyme (250 pM) to 180 μL 200 μM nitrocefin. Absorbance at 490 nm was measured continuously in 4-s intervals for 2 min with a Spectramax plate reader (Molecular Devices). The background absorbance in the absence of the enzyme was subtracted. Reaction rates were converted to percent activity of the uninhibited enzyme control.

NMR Spectroscopy. All NMR spectra were acquired at 37 °C with a 600 MHz NMR instrument with a Bruker AVANCE III console and a triple-resonance cryogenic probe. The NMR samples were prepared in 100 mM sodium phosphate buffer (pH 7.0) with 5% D_2O. To suppress or eliminate signals from covalently bound compound and protein, a Carr–Purcell–Meiboom–Gill spin echo train sequence (1, 2) was introduced before the water suppression and the acquisition period. A total of a 236-ms Carr–Purcell–Meiboom–Gill period was used for all compound-observed NMR experiments. Water suppression was achieved by the excitation sculpting scheme (3), and the water-selective 180° Sinc shape pulse was 3 ms long. The data were collected with a 9,157 Hz sweep width and 1.5-s relaxation delay. Eighty scans were recorded for each experiment, which resulted in 5 min recording time per spectrum. The data were multiplied by an exponential function with 3 Hz line broadening before Fourier transformation.

Equilibration of Acyl-Enzyme with Free Enzyme: Enzyme Activity Measurement. TEM-1 acyl-enzyme (1 μM) was prepared as described in *Materials and Methods*. To assess initial enzyme activity, each acyl-enzyme concentration was diluted to 120 pM TEM-1, and activity was assayed immediately by adding 20 μL 120 pM enzyme to 180 μL 400 μM nitrocefin and measuring 490 nm absorbance. Initial reaction rates during the first 2 min were converted to percent activity with respect to the uninhibited enzyme control.

Protein MS. Intact protein samples were analyzed by positive ion electrospray ionization (ESI)-MS on a QSTAR Pulsar i mass spectrometer (Applied Biosystems) equipped with a Turbo Ion Spray source. Protein mass spectra were deconvoluted using Analyst QS software (version 1.1). Formic acid and methanol were added to samples to reach final concentrations of 0.1% and 5% by volume, respectively. Samples were injected onto a Michrom Microtrap cartridge, washed with 2 mL buffer A (0.1% formic acid, 5% methanol), and eluted with buffer B (0.1% formic acid, 90% acetonitrile) into the mass spectrometer. Relative peak intensities (area percentages) were calculated from total peak heights or areas of apo- and acyl-protein, and they included minor contributions from cation adducts and a small, ionization-induced peak corresponding to an 80-Da loss from the acyl-enzyme. Lowering the declustering potential to 40 V, with spray voltage at 5,000 V and source temperature at 200 °C, minimized the relative abundance of the 80-Da loss from the acyl enzyme. A similar observation was made in the work by Stachyra et al. (4).

To confirm the covalent nature of the acyl-enzyme complex (Fig. S2), acylated TEM-1 (1 μM) in assay buffer was treated with guanidine HCl to a final concentration of 6.5 M (pH 5.5) and incubated at 80 °C for 30 min. The sample was desalted by an ultrafiltration cartridge as described above to remove large amounts of denaturant followed by Macrotrap cartridge desalting before MS analysis. The MS spectrum revealed exclusively acyl-TEM-1 with adducts corresponding to oxidation (+16 and +32), which was expected for chemical denaturation.

To assess rearrangements of avibactam and clavulanic acid in the presence of TEM-1 (Fig. S3*B*), TEM-1 (1 μM) was incubated with either compound at 5 and 250 μM concentration at 37 °C for 30 min. After desalting with a macrotrap cartridge as described above, only acylated TEM-1 was observed with avibactam at either concentration. With 250 μM clavulanic acid, a series of adducts (+51, +70, +87, and +155) were observed that were identical to masses observed previously (5).

For the acyl-enzyme exchange from TEM-1 to other enzymes (Fig. 6*C* and Fig. S5), the acylated β-lactamases CTX-M-15, KPC-2, and *Enterobacter cloacae* P99 were prepared through incubation of 1 μM enzyme and 1 μM avibactam to yield initially 100% acylation. *Pseudomonas aeruginosa* AmpC was incubated with 5 μM avibactam and yielded a 77% initial acylation. Each donor acyl-enzyme was combined in pairwise fashion with 1 μM apo-TEM-1 to observe the mass transfer of avibactam from the test β-lactamase to TEM-1.

Liquid Chromatography Tandem MS Quantification of Avibactam. Liquid chromatography tandem MS was performed on a Shimadzu HPLC system connected to an ABSciex QTrap 5500 mass spectrometer equipped with a turbo ion-spray ionization source operated in the negative mode. To prepare samples for liquid chromatography tandem MS analysis, 160 μL acetonitrile spiked with 30 nM internal standard were mixed with 40 μL incubated dilution samples and centrifuged at 14,000 rpm for 3 min; 10 μL supernatant were injected on a 3 × 50-mm Luna (Phenomenex) HILIC column with 3-μm particle size. The mobile phase was 0.1% (vol/vol) formic acid in water (A) and 0.1% (vol/vol) formic acid in acetonitrile (B). The separation was performed by a 0.5-min linear gradient from 5% to 20% A and held at 20% A for 3 min at a flow rate of 0.4 mL/min. Avibactam was monitored by quantifying the multiple reaction monitoring peak area. The area ratio of avibactam to the area of the internal standard was used to calculate the concentration of avibactam. A set of avibactam calibration standards was prepared in parallel to the dilution samples and used to quantify the unknowns. The ion transition of avibactam was 264.2/96.0 amu. Mass spectrometer source conditions of curtain gas, gas 1, and gas 2 were set at 30, 40, and 50 psi, respectively. The ion spray voltage was 4,000, and the temperature was 400 °C. The optimum voltage settings for the declustering potential, the collision energy, and the collision exit potential were 60, 34, and 9 V, respectively.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) was performed on a VP-ITC instrument (GE Healthcare) at 37 °C using 100 mM sodium phosphate (pH 7.0) as buffer for all solutions. Phenylethylboronic acid (CAS# 34420–17-2) was obtained from Sigma-Aldrich. Protein was extensively dialyzed against this buffer at 4 °C using 10,000 M_r cutoff cassettes (Thermo Scientific). At the conclusion of dialysis, protein was recovered,

and the dialysis buffer was retained for subsequent ITC titrations as well as preparation of phenylethylboronic acid and avibactam solutions. The protein concentration was determined in 6 M guanidine chloride solution using a calculated extinction coefficient of 28,020 $M^{-1}cm^{-1}$ at 280 nm, as previously described (6). Because of the low affinity of phenylethylboronic acid for TEM-1, binding studies were performed with a low concentration of protein relative to the dissociation constant for the binding interaction. Consequently, the titrations were designed to attain, by the end of the experiment, a large ratio of total ligand to both protein and the dissociation constant (~100-fold) as described (7) to assure adequate definition of the affinity constant. For both phenylethylboronic acid (direct titration) and avibactam (competitive titration) affinity measurements, three replicate measurements were performed. Results reported are the mean values of the replicates, and the error is 2 SDs.

For phenylethylboronic acid affinity determinations, experiments were conducted with 30 μM protein in the cell and 20 mM phenylethylboronic acid in the syringe. A total of 55 injections were made, the first injection being 2 μL and the rest of the injections being 4 μL. Injections were spaced 270 s apart. For the avibactam competitive binding titrations, the cell contained 30 μM protein and 10 mM phenylethylboronic acid, and the syringe contained 500 μM avibactam and 10 mM phenylethylboronic acid in phosphate buffer. Injections were spaced 360 s apart. Data were analyzed using the Origin fitting software package (version 7) as provided by the manufacturer. Direct binding experiments were analyzed as a single-site model, with the stoichiometry of binding defined as being one. Competitive binding experiments were analyzed using the method previously described (8): fixing the stoichiometry of phenylethylboronic acid binding as one and the heat of avibactam binding as −24.4 kcal/mole. This value for the heat of avibactam binding was determined by making 16 injections of 10 μL each of 500 μM avibactam (syringe) into 30 μM TEM-1 (cell) at 37 °C.

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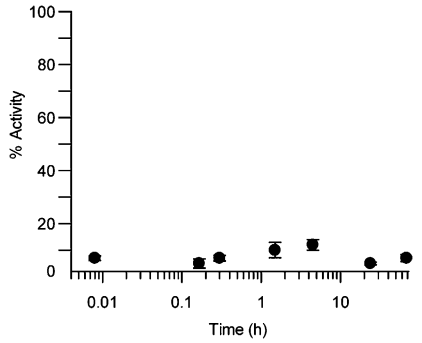

Fig. S1. Stability of acyl-enzyme sampled over time; 1 μM acyl-TEM-1 was assayed up to 68 h for initial enzyme activity. Errors bars shown are ±SEM from two or three measurements.

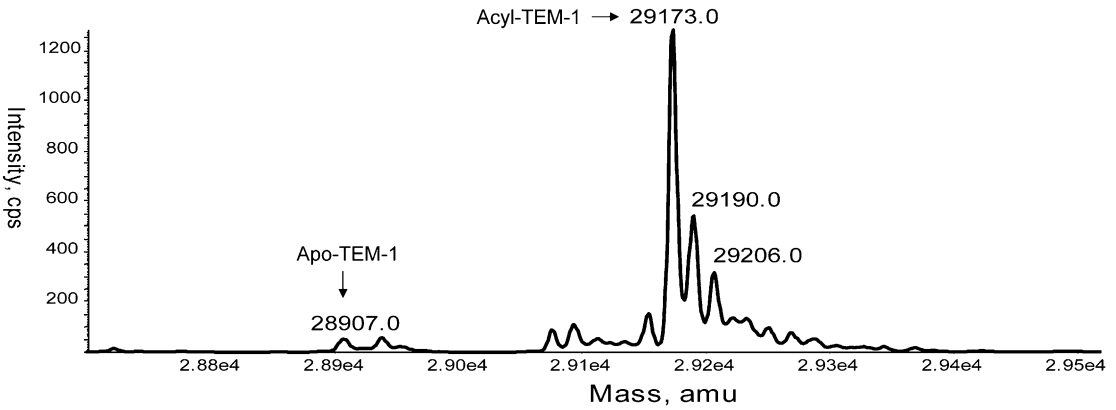

Fig. S2. Stability of acyl-enzyme to denaturation. Mass spectrum of acyl-TEM-1 after exposure to 6.5 M guanidine chloride at 80 °C for 30 min. Expected mass of apo-TEM-1 is 28,907 amu, and expected mass of acyl-TEM-1 is 29,172 amu.

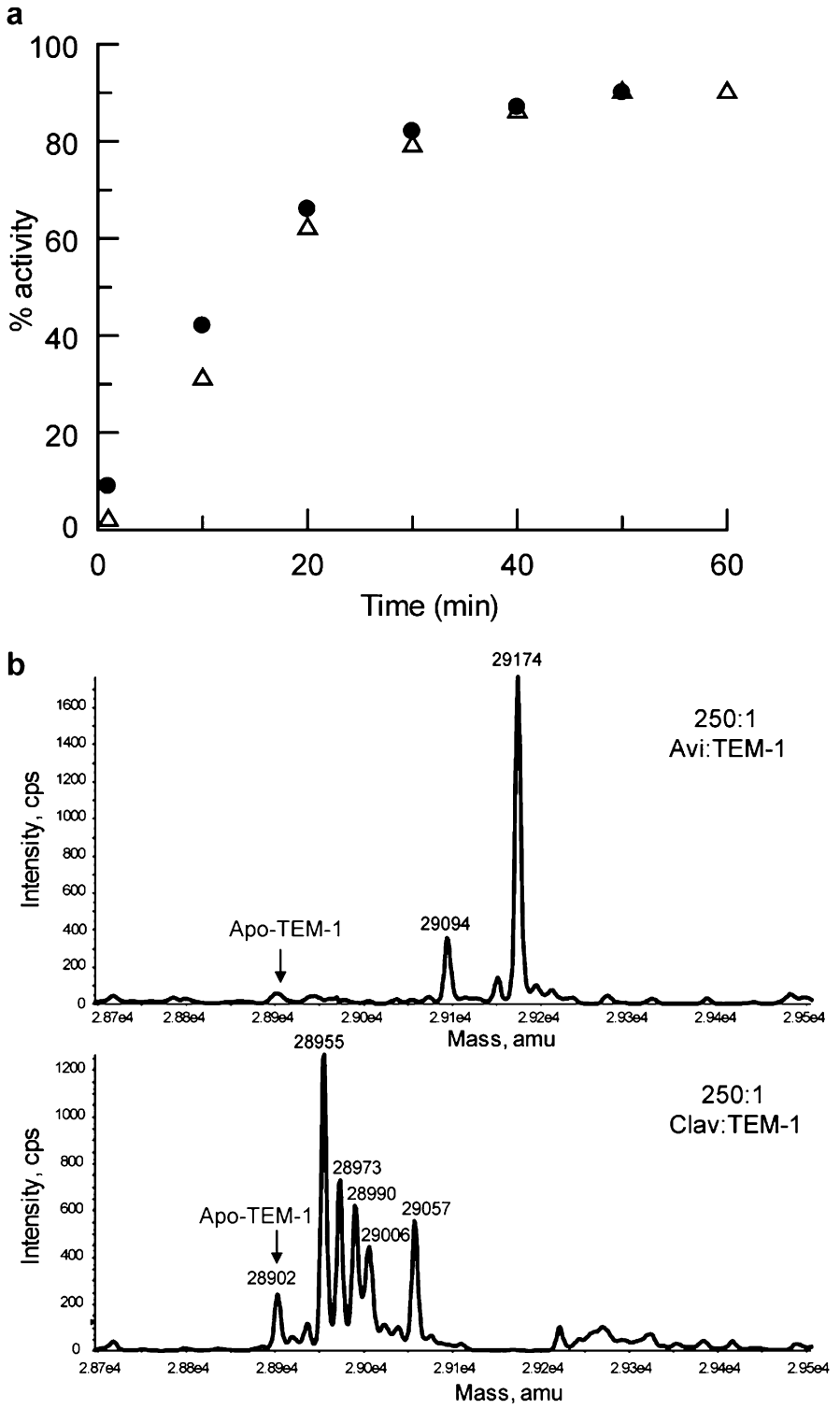

Fig. S3. Incubations at high inhibitor concentrations. (*A*) Return of activity after incubation of 1 μM TEM-1 with avibactam concentrations of 5 (filled circles) and 250 μM (open triangles). (*B*) Mass spectra after incubation of 1 μM TEM-1 with 250 μM avibactam (*Upper*) and 250 μM clavulanic acid (*Lower*). Expected masses: apo-TEM-1, 28,907 amu; avibactam acyl-TEM-1, 29,172 amu; clavulanic acid acyl-TEM-1, 29,106 amu.

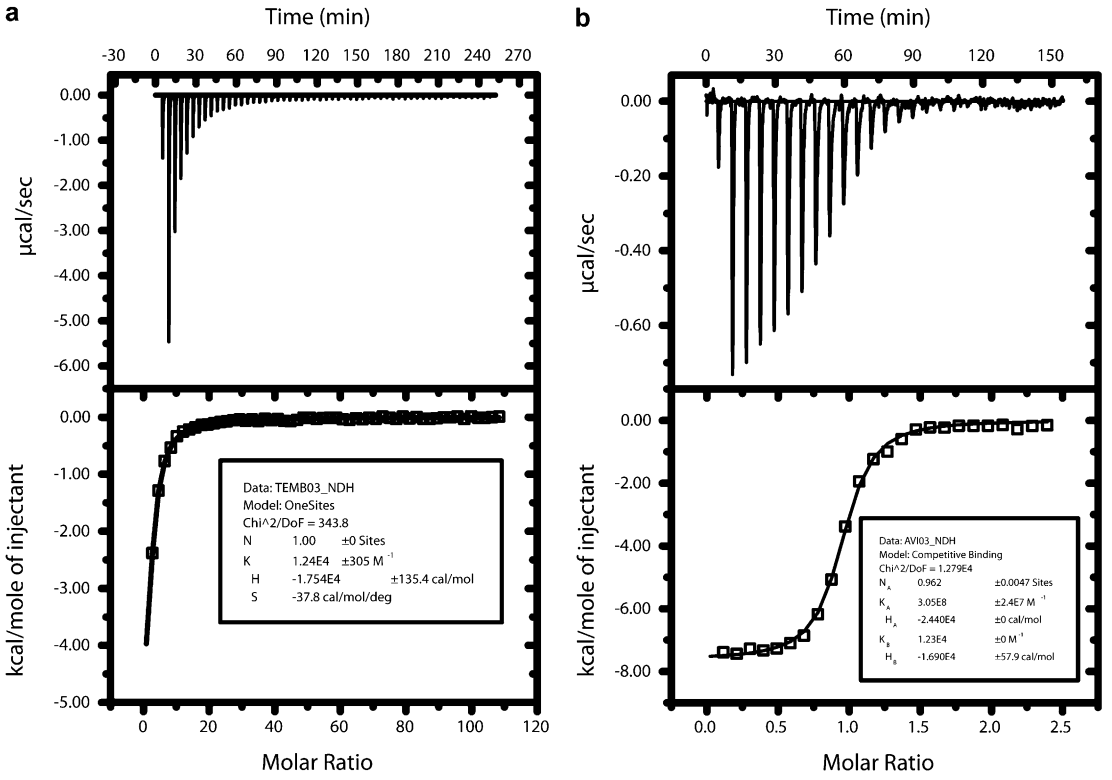

Fig. S4. Isothermal titration calorimetry to determine the dissociation constant for the TEM-1 avibactam complex by competition with phenylethylboronic acid. Titrations were performed in triplicate with injection protocols and analyte concentrations as described in *SI Materials and Methods*. Results reported in the subpanel descriptions below for parameters determined represent the mean value ± 2 SD from the replicates. (*A*) Representative titration of phenylethylboronic acid into TEM-1. Average parameter results obtained are $K_a = (12.3 \pm 0.2) \times 10^3$ M^{-1} equivalent to $K_d = (82 \pm 2)$ µM, $\Delta H = (-17.3 \pm 0.6)$ kcal mol^{-1}, and $-T\Delta S = (11.5 \pm 0.6)$ kcal mol^{-1}. (*B*) Representative titration of avibactim displacing phenylethylboronic acid from TEM-1. Average parameter results obtained for avibactam binding are $n = (0.97 \pm 0.03)$ and $K_a = (3.0 \pm 0.4) \times 10^8$ M^{-1} equivalent to $K_d = (3.3 \pm 0.4)$ nM. Additionally, the enthalpy of phenylethylboronic acid binding was determined as $\Delta H = (-16.8 \pm 0.2)$ kcal mol^{-1}.

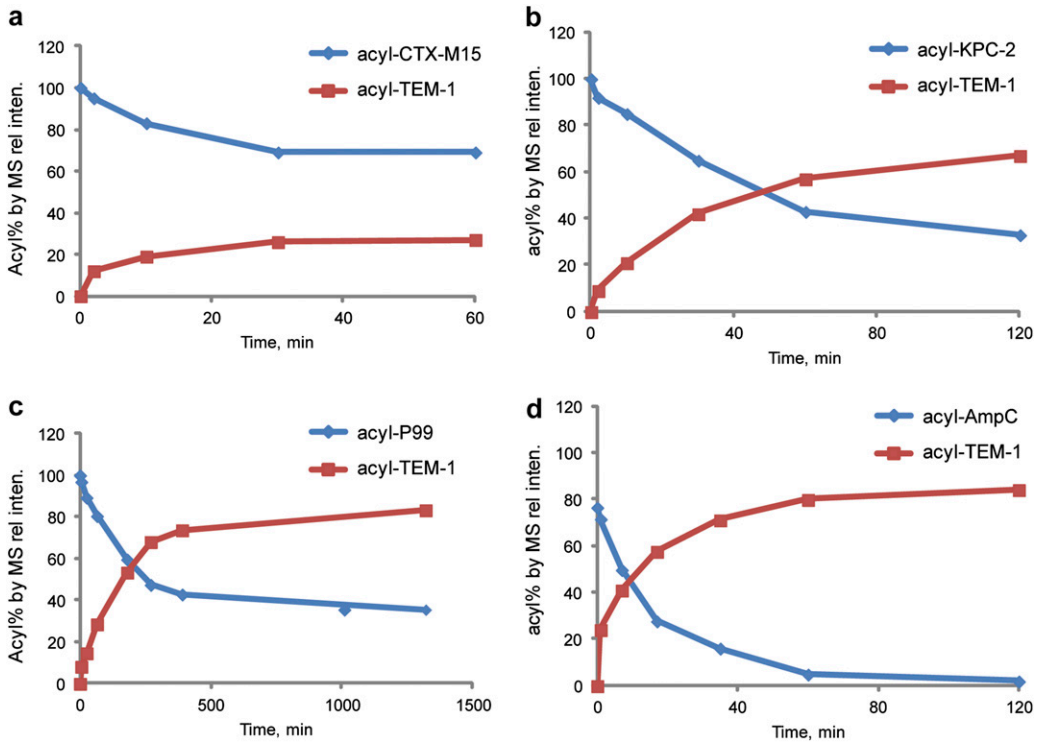

Fig. S5. Time courses of acyl-enzyme exchange to apo-TEM-1 from donor acylated β-lactamases. (*A*) CTX-M-15. (*B*) KPC-2. (*C*) *E. cloacae* P99. (*D*) *P. aeruginosa* AmpC.