A Specific Mechanism of Nonspecific Inhibition

Susan L. McGovern,[†] Brian T. Helfand,[‡] Brian Feng,[§] and Brian K. Shoichet*[,§]

Department of Molecular Pharmacology and Biological Chemistry and Department of Cell and Molecular Biology, Northwestern University, 303 East Chicago Avenue, Chicago, Illinois 60611, and Department of Pharmaceutical Chemistry, Genentech Hall, University of California−San Francisco, 600 16th Street, San Francisco, California 94143

Received June 3, 2003

Promiscuous small molecules plague screening libraries and hit lists. Previous work has found that several nonspecific compounds form submicrometer aggregates, and it has been suggested that this aggregate species is responsible for the inhibition of many different enzymes. It is not understood how aggregates inhibit their targets. To address this question, biophysical, kinetic, and microscopy methods were used to study the interaction of promiscuous, aggregate-forming inhibitors with model proteins. By use of centrifugation and gel electrophoresis, aggregates and protein were found to directly interact. This is consistent with a subsequent observation from confocal fluorescence microscopy that aggregates concentrate green fluorescent protein. β-Lactamase mutants with increased or decreased thermodynamic stability relative to wild-type enzyme were equally inhibited by an aggregate-forming compound, suggesting that denaturation by unfolding was not the primary mechanism of interaction. Instead, visualization by electron microscopy revealed that enzyme associates with the surface of inhibitor aggregates. This association could be reversed or prevented by the addition of Triton X-100. These observations suggest that the aggregates formed by promiscuous compounds reversibly sequester enzyme, resulting in apparent inhibition. They also suggest a simple method to identify or reverse the action of aggregate-based inhibitors, which appear to be widespread.

Introduction

Some small molecules inhibit many different enzymes. These promiscuous compounds include hits from virtual and high-throughput screening[1−5] as well as some biological reagents.[6−9] Their lack of specificity complicates their use as leads for drug design or as pharmacological tools for dissecting biological systems, and much work has been devoted to identifying and understanding these enigmatic molecules.[10−16]

Consequently, several mechanisms have been proposed for compound promiscuity. Some molecules covalently react with functional groups in proteins, leading to irreversible inhibition of target enzymes.[3] Other inhibitors contain "privileged" substructures that can bind several different members of a protein family.[10] Still other molecules are known to interfere with colorimetric or fluorimetric detection methods used in screening assays, thereby creating the impression of inhibition via experimental artifact.[10]

Even after considering these mechanisms, there remained a population of nonspecific enzyme inhibitors that defied explanation. Previously, we found that apparently unrelated promiscuous compounds shared several peculiar properties: time-dependent behavior, steep inhibition curves, and sensitivity to enzyme concentration and ionic strength. To account for this behavior, we proposed that these molecules shared the ability to form aggregates in solution, and the aggregate species was responsible for inhibiting various enzymes.[17] This hypothesis was supported by light scattering and electron microscopy experiments in which promiscuous compounds were observed to form particles of 100−1000 nm diameter.[17,18]

The aggregation model left several questions unanswered. For instance, how do aggregates interact with the enzymes they inhibit? Even more fundamentally, do aggregates and enzyme interact at all? Once aggregate-mediated inhibition has occurred, can it be reversed?

Here, we explore these questions using centrifugation and electrophoresis, enzyme kinetics, light scattering, confocal fluorescence microscopy, and transmission electron microscopy. Results from these experiments suggest a model for aggregate−enzyme association, as well as a simple assay for differentiating aggregate-based inhibitors from well-behaved inhibitors.

Results

If aggregates formed by a promiscuous compound are responsible for enzyme inhibition, removal of aggregates from a solution of the compound should decrease inhibition. To test this prediction, we centrifuged a solution of tetraiodophenolphthalein (I4PTH), a promiscuous, aggregate-forming compound with an IC_{50} value of 3 μM against the enzyme β-lactamase.[17] Dynamic light scattering (DLS) analysis of the resulting supernatant revealed that centrifugation for 30 min at 15682*g* was sufficient to pellet out many of the particles from the solution of I4PTH (Figure 1). The apparent IC_{50} value of the supernatant against β-lactamase was 70 μM. Since the IC_{50} value of I4PTH increased (worsened) from

* To whom correspondence should be addressed. E-mail: shoichet@cgl.ucsf.edu. Phone: 415-514-4126. Fax: 415-502-1411.
[†] Department of Molecular Pharmacology and Biological Chemistry, Northwestern University.
[‡] Department of Cell and Molecular Biology, Northwestern University.
[§] University of California−San Francisco.

10.1021/jm030266r CCC: $25.00
Published on Web 08/22/2003

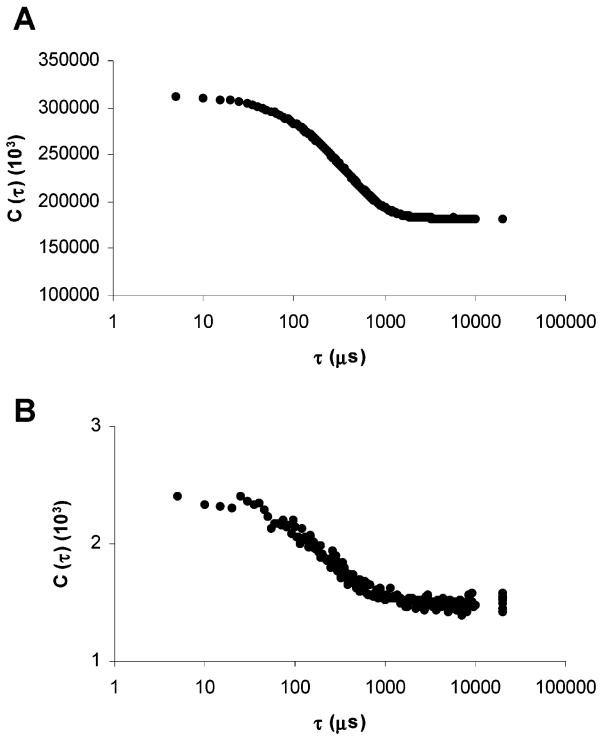


Figure 1. Representative autocorrelation functions from DLS before and after centrifugation of promiscuous inhibitors. (A) Autocorrelation function of 100 μM I4PTH before centrifugation. The average intensity was 366 kcps (kilocounts per second), and the IC_{50} vs β-lactamase was 3 μM. (B) Autocorrelation function of 100 μM I4PTH after centrifugation. The average intensity was 1 kcps, and the IC_{50} vs β-lactamase was 70 μM. Laser power and integration times were comparable for all DLS experiments.

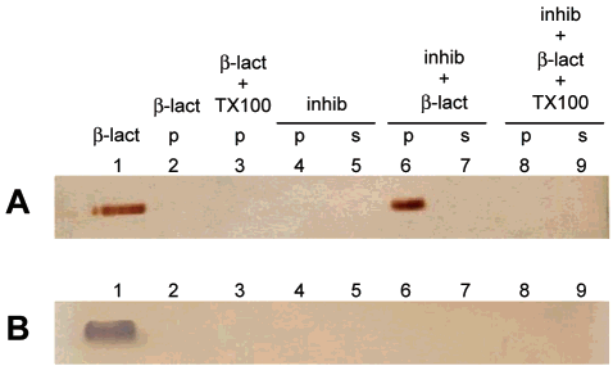


Figure 2. SDS−PAGE and silver-stain analysis of supernatants and pellets from centrifugation of β-lactamase in the presence or absence of inhibitor with and without Triton X-100. (A) The inhibitor is tetraiodophenolphthalein (I4PTH), a promiscuous, aggregate-forming compound.[17] (B) The inhibitor is 1-(2-thienylacetylamino)-1-(3-carboxyphenyl)methylboronic acid, a specific, nonaggregating inhibitor of β-lactamase.[19] For both gels, lane 1 is 3000 ng of β-lactamase loaded directly onto the gel, lane 2 is the pellet from centrifugation of β-lactamase alone, lane 3 is the pellet from centrifugation of β-lactamase with 0.01% Triton X-100, lanes 4 and 5 are the pellet and supernatant from centrifugation of 100 μM inhibitor alone, lanes 6 and 7 are the pellet and supernatant from centrifugation of 100 μM inhibitor with β-lactamase, and lanes 8 and 9 are the pellet and supernatant from centrifugation of 100 μM inhibitor with β-lactamase and 0.01% Triton X-100. A 3000 ng sample of β-lactamase was used for each centrifugation experiment. Abbreviations are the following: s, supernatant; p, pellet; β-lact, β-lactamase; TX100, Triton X-100; inhib, inhibitor.

3 μM before centrifugation to 70 μM after centrifugation, the removal of aggregates by centrifugation resulted in a decrease in inhibition. Similar results were obtained with other aggregate-forming compounds; in each case, the supernatant had an increased (worse) IC_{50} value against β-lactamase compared to the solution before centrifugation (data not shown). Conversely, the concentration of well-behaved, nonaggregating molecules, such as 8-anilino-1-naphthalenesulfonic acid (ANS), a dye that does not inhibit β-lactamase, and moxalactam, a well-studied covalent inhibitor of that enzyme, did not change after centrifugation.

To determine if aggregates and enzymes directly interacted, we next asked if enzyme could cosediment from solution with aggregates. We repeated the centrifugation experiment, adding β-lactamase to the solution in the presence and absence of aggregates. This time, any resulting pellet was resuspended and analyzed by SDS−PAGE and silver stain. Centrifugation of β-lactamase alone for 30 min at 15682*g* did not pellet out the enzyme, as expected (Figure 2A, lane 2). On the other hand, centrifugation of β-lactamase in the presence of I4PTH aggregates concentrated the enzyme in the pellet (Figure 2A, lane 6). Similar results were obtained with other promiscuous inhibitors (data not shown). In control experiments, 1-(2-thienylacetylamino)-1-(3-carboxyphenyl)methylboronic acid, a specific, nonaggregating, competitive inhibitor of β-lactamase (K_i = 1 nM),[19] did not concentrate β-lactamase in the pellet (Figure 2B, lane 6). The ability of β-lactamase to cosediment with aggregates is consistent with a direct association between these species.

If aggregates and enzyme directly interact, how do they do so? We considered two models of aggregate−enzymes interaction: (1) aggregates could absorb enzyme; (2) aggregates could adsorb enzyme.[17] To explore these possibilities, we used transmission electron microscopy (TEM) to observe the interaction of I4PTH aggregates with β-galactosidase, an enzyme that is inhibited by I4PTH[17] and is large enough to be directly visualized by TEM (Figure 3B). In a mixture of 100 μM I4PTH and 0.1 mg/mL β-galactosidase, enzyme was found on the surface of aggregates (parts C and D of Figure 3), often in nests of multiple aggregates binding many molecules of β-galactosidase. Visual inspection of several hundred enzyme molecules revealed that approximately 90% were aggregate-bound; only 10% were free.

To further investigate the interaction of aggregates with protein, we used confocal fluorescence microscopy to study the interaction of I4PTH aggregates with green fluorescent protein (GFP). GFP alone produced a diffuse field of fluorescence (Figure 4A). In the presence of I4PTH, numerous discrete accumulations of GFP were observed (Figure 4B). Given that the resolution of this method was 0.2 μm and that these focal points were about 1 μm in diameter, these bright spots likely correspond to nests of aggregates interacting with multiple molecules of GFP, as observed in corresponding TEM images with GFP and I4PTH (Figure 4E) and in similar images with β-galactosidase and I4PTH (parts C and D of Figure 3).

The cosedimentation and microscopy experiments are consistent with direct interaction between enzyme and

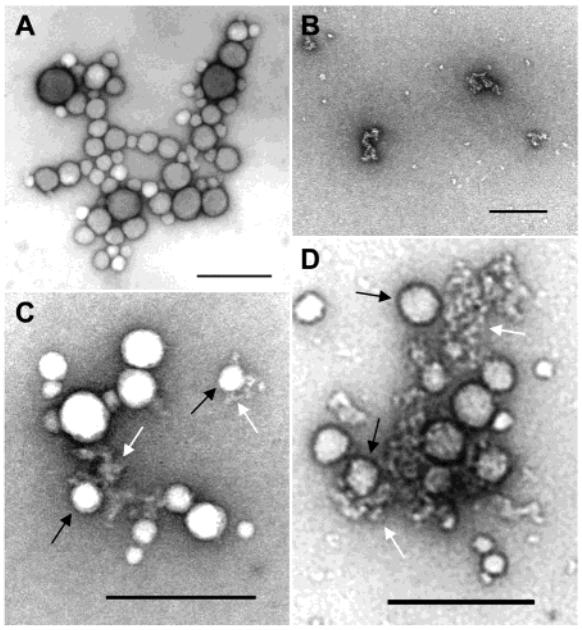


Figure 3. Visualization of I4PTH aggregates and β-galactosidase molecules by TEM: (A) 100 μM I4PTH alone; (B) 0.1 mg/mL β-galactosidase alone; (C, D) 100 μM I4PTH with 0.1 mg/mL β-galactosidase. Representative I4PTH aggregates are marked with black arrows, and β-galactosidase molecules are marked with white arrows. The bar represents 200 nm.

aggregate, perhaps favoring a surface-based mechanism (adsorption). They do not reveal if the enzyme is denatured or simply sequestered or if the inhibition is reversible. To investigate denaturation by unfolding, we compared aggregate-mediated inhibition of two stability mutants of TEM-1 β-lactamase: M182T, which is 2.7 kcal/mol (6.2 °C) more stable than wild-type TEM-1,[20] and G238A, which is 1.8 kcal/mol (4.1 °C) less stable than wild-type TEM-1[20] (Table 1). Both mutant enzymes were assayed at the same concentration. The IC_{50} values for the enzymes were the same, within error, at both 24 and 42 °C. This suggests that if the aggregates are denaturing the enzyme, it is not through unfolding. Consistent with this view, GFP retained fluorescence in the discrete accumulations of this protein observed in the presence of aggregates (Figure 4B), suggesting that the protein maintained its native structure while interacting with aggregate particles.

Table 1. Inhibition of TEM-1 β-Lactamase Stability Mutants by I4PTH

			IC_{50} (μM) of I4PTH	
mutant	$\Delta\Delta G$ [a] (kcal/mol)	ΔT_m [a] (°C)	24 °C	42 °C
G238A	−1.8	−4.1	3	7
M182T	+2.7	+6.2	3	5

[a] Relative to wild-type TEM-1 β-lactamase.[20]

This made us wonder if inhibition might be reversible by disruption of the aggregates. We turned to nonionic detergents, such as saponin, digitonin, and Triton X-100, which we had found could diminish aggregate-based inhibition. For instance, the addition of 0.01% Triton X-100 decreased inhibition of β-lactamase by 14 aggregate-forming, promiscuous compounds but not by benzo[*b*]thiophene-2-boronic acid (BZBTH2B), a specific and well-behaved inhibitor of β-lactamase (Table 2). Similar results were obtained with 0.001% Triton X-100 (data not shown).

To explore whether Triton X-100 could reverse aggregate-based inhibition, four cuvettes with 1 nM β-lactamase each were prepared in 50 mM potassium phos-

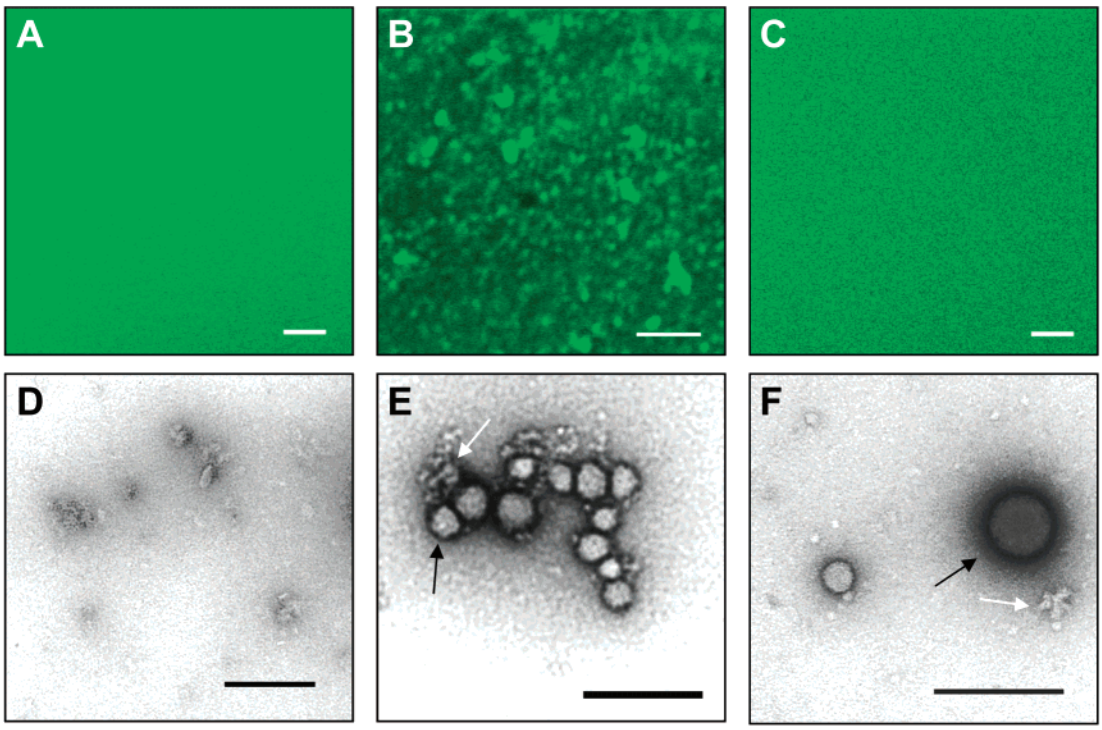
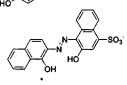
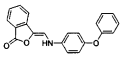
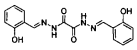
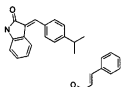
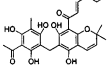
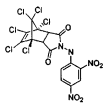
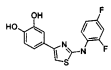
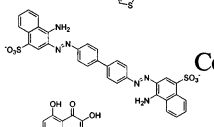
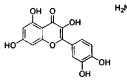
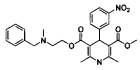


Figure 4. Visualization of I4PTH aggregates and GFP molecules by confocal fluorescence microscopy (A−C) and TEM (D−F): (A) 0.33 mg/mL GFP alone; (B) 0.25 mg/mL GFP with 500 μM I4PTH; (C) 0.25 mg/mL GFP with 500 μM I4PTH and 0.0075% Triton X-100; (D) 0.5 mg/mL GFP alone; (E) 0.1 mg/mL GFP with 100 μM I4PTH; (F) 0.1 mg/mL GFP with 100 μM I4PTH and 0.001% Triton X-100. Representative I4PTH aggregates are marked with black arrows, GFP molecules are marked with white arrows. The bar represents 5 μm in panels A−C, and the bar represents 200 nm in panels D−F.

Table 2. Effect of 0.01% Triton X-100 on Inhibition of β-Lactamase by Known Aggregate-Forming Inhibitors

Compound	Compound Name	IC_{50} vs. β-lactamase (μM)	Assay Conc. (μM)	% β-lactamase inhibition	
				No TX100	+ 0.01% TX100
	BZBTH2B [a]	0.2[17]	0.6	87	86
	I4PTH	3[17]	10	78	3
	K-252c	8[18]	20	80	17
	MFCD00093929[c]	5[17]	20	91	10
	MFCD00003934[c]	10[17]	30	68	10
	MFCD00139657[c]	5[17]	20	99	26
	MFCD00187101[c]	10[17]	30	96	39
	MFCD00118155[c]	6[17]	20	95	27
	Rottlerin	1.2[18]	5	90	5
	MFCD00225114[c]	3[17]	10	73	1
	MFCD00955233[c]	18[17]	60	84	14
	Congo Red	4[17]	10	92	20
	Quercetin	4[17,18]	25	68	25
	Sulconazole	14[b]	30	93	11
	Nicardipine	20[b]	100	81	33

[a] A specific inhibitor of β-lactamase.[21] [b] Unpublished observations. [c] Available Chemicals Directory (ACD) registry number. See Materials for full name.

phate (KPi) buffer. Two of these cuvettes also contained 10 μM I4PTH (thick lines in Figure 5A); the other two had no inhibitor (thin lines in Figure 5A). After 5 min of incubation, the β-lactamase reaction was initiated in each cuvette by the addition of substrate (time is 0 s in Figure 5A); the enzyme was 75% inhibited by the aggregating molecules, as expected. After approximately 100 s of hydrolysis, 10 μL of 1% Triton X-100 was added to each cuvette for a final concentration of 0.01% Triton X-100 in a total reaction volume of 1 mL. Almost immediately, β-lactamase activity in the cuvettes with I4PTH returned to approximately 70% of that of the uninhibited control (Figure 5A). β-Lactamase activity in the control cuvettes was unaffected by the addition of Triton X-100. Similar results were obtained with 5 μM rottlerin (Figure 5B) and other aggregate-forming, promiscuous enzyme inhibitors (data not shown). As a negative control, the same experiment was performed with 0.6 μM BZBTH2B, a specific and well-behaved inhibitor of β-lactamase; upon detergent addition, activity did not return to the cuvettes containing this inhibitor (Figure 5C). These observations suggest that the mechanism of aggregate-based inhibition is reversible sequestration of enzyme.

The effect of Triton X-100 was further investigated by microscopy, centrifugation, and light scattering. Triton X-100 at 0.001% did not affect the gross appearance of I4PTH aggregates or β-galactosidase alone (parts A and B of Figure 6). However, when I4PTH aggregates and β-galactosidase were mixed with 0.001% Triton X-100, β-galactosidase was no longer found on the surface of aggregates (Figure 6C). Visual inspection

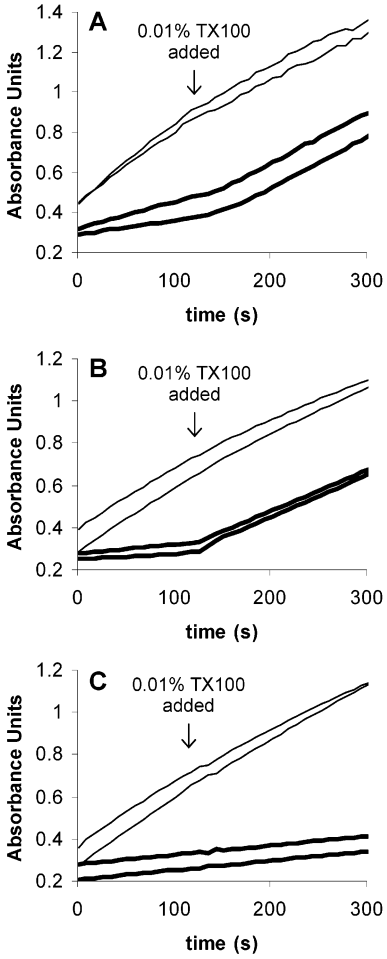


Figure 5. Effect of 0.01% Triton X-100 added during a β-lactamase inhibition assay. (A) Inhibitor is 10 μM I4PTH. (B) Inhibitor is 5 μM rottlerin. (C) Inhibitor is 0.6 μM BZBTH2B, a specific β-lactamase inhibitor.[21] In all panels, thick lines (—) denote reactions containing inhibitor and thin lines (−) denote reactions containing DMSO control. TX100 is Triton X-100.

of hundreds of β-galactosidase molecules over several different fields revealed that only about 1% of the enzyme was bound to aggregate; the remainder was free. This contrasted markedly with the absence of detergent, in which case approximately 90% of the enzyme was bound to aggregates (parts C and D of Figure 3). Similarly, GFP did not bind I4PTH aggregates in the presence of 0.001% Triton X-100 (Figure 4F). Consistent with these observations, a mixture of aggregates, GFP, and Triton X-100 visualized with the confocal microscope produced diffuse fluorescence (Figure 4C), similar to the appearance of GFP alone (Figure 4A).

To determine the effect of further increasing the concentration of detergent, a mixture of 10 μM I4PTH with 0.01% Triton X-100 was analyzed by dynamic light scattering. This concentration of detergent resulted in a low-intensity autocorrelation function with weak decay (Figure 7B), compared to that in the absence of detergent (Figure 7A). Similar results were obtained with seven other promiscuous, aggregate-forming compounds (Table 3); the scattering intensity of each inhibitor decreased in the presence of 0.01% Triton X-100, consistent with a decrease in the concentration of aggregates. In contrast, aggregates were still present after the addition of 0.001% Triton X-100 (data not shown), even though this concentration of detergent was sufficient to prevent enzyme inhibition (data not shown).

Taken together, the kinetic, microscopic, and light scattering results suggest that low concentrations of Triton X-100 disrupt the aggregate−protein interaction and prevent inhibition (Figures 4 and 6; data not shown). At higher concentrations of detergent, the aggregates themselves are destroyed (Figure 7 and Table 3).

Most of the promiscuous, aggregate-forming inhibitors studied were initially prepared as 10 mM stocks in dimethyl sulfoxide (DMSO). Aliquots from this stock were then typically diluted in 50 mM KPi buffer for subsequent inhibition and light scattering assays. It is possible that the aggregate species represents a metastable state resulting from transfer of the compound from an organic solvent into an aqueous environment. If so, aggregate formation and enzyme inhibition should not occur in a pure aqueous solution of inhibitor. To investigate this hypothesis, solutions of four promiscuous, aggregate-forming compounds were each prepared in 50 mM KPi, without DMSO, and assayed for inhibition of β-lactamase (Table 4). Each compound retained the ability to inhibit the enzyme, with potencies similar to those obtained from DMSO stocks at the same concentration. However, the apparent solubility of the inhibitors was much less in the absence of DMSO; for instance, nicardipine was easily miscible at 60 μM in 50 mM KPi when diluted from a DMSO stock, but it was only soluble up to 13.5 μM in the absence of DMSO. These preliminary observations suggest that although DMSO affects apparent compound solubility and can thereby increase inhibition, it is not required for aggregate-mediated enzyme inhibition to occur.

Discussion

Inhibition by promiscuous, aggregate-forming compounds results from a reversible physical association between aggregates and enzymes. Electron microscopy suggests that this interaction occurs through protein adsorption onto the surface of aggregates, although absorption certainly cannot be excluded. It appears that aggregates associate with and sequester enzyme molecules, thereby inhibiting them.

An adsorption mechanism is consistent with our previous observation that aggregate-based inhibitors are sensitive to enzyme concentration.[17] If the aggregate surface were saturated with enzyme, any additional enzyme added would be free in solution. Similarly, it is consistent with the observation that the addition of bovine serum albumin decreases the potency of aggregate-based inhibitors;[17] albumin could also saturate aggregate surface and leave free enzyme in solution. This mechanism is also compatible with the noncompetitive nature of these inhibitors[17] because enzyme sequestration would reduce the number of binding sites available for substrate. Perhaps most importantly, the mechanism is consistent with the promiscuity of the aggregates because adsorption between two large surfaces would have only gross electrostatic and steric selectivities.

Intriguingly, inhibition can be prevented by nonionic detergents and reversed by Triton X-100. Biophysical,

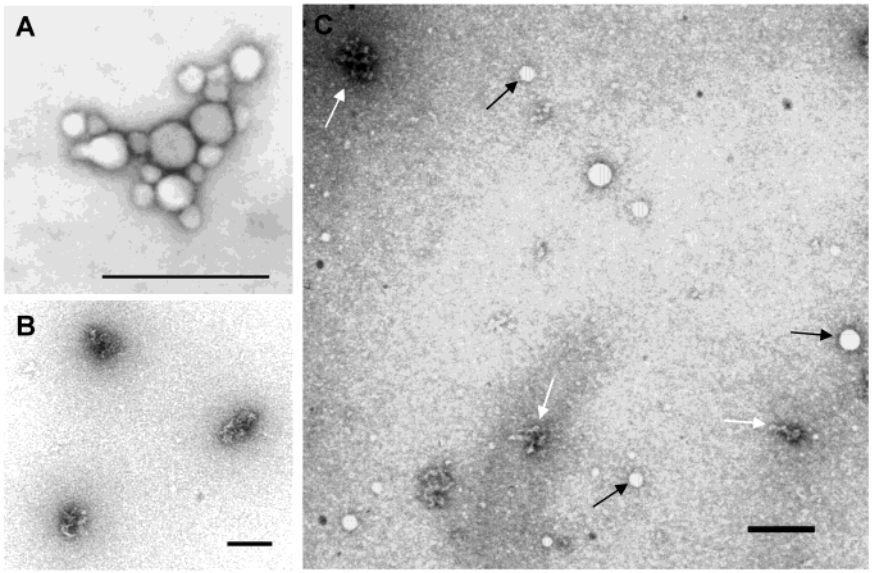


Figure 6. Visualization of I4PTH aggregates and β-galactosidase molecules with Triton X-100 by TEM: (A) 100 μM I4PTH with 0.001% Triton X-100; (B) 0.1 mg/mL β-galactosidase with 0.001% Triton X-100; (C) 100 μM I4PTH, 0.1 mg/mL β-galactosidase, and 0.001% Triton X-100. Representative I4PTH aggregates are marked with black arrows, and β-galactosidase molecules are marked with white arrows. The bar represents 200 nm.

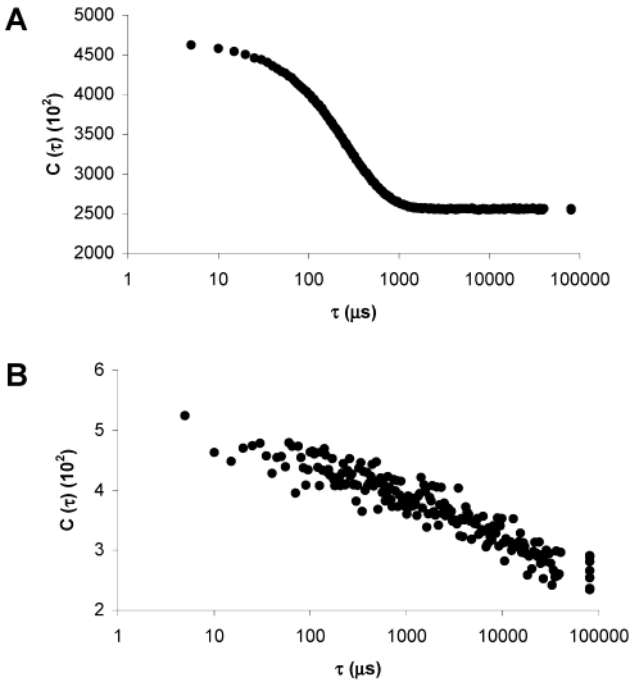


Figure 7. Representative autocorrelation functions from DLS on aggregate-forming inhibitors with and without Triton X-100: (A) 10 μM I4PTH alone; (B) 10 μM I4PTH with 0.01% Triton X-100.

microscopic, and enzymatic techniques suggest the following mechanism of action. Low concentrations of Triton X-100 prevent the aggregate−enzyme interaction, although many aggregate particles remain in solution. At higher concentrations of detergent, aggregate particles themselves disintegrate. Concentrations of Triton X-100 up to 0.01% do not appear to affect the activity of the model enzymes studied here or that of classically behaved inhibitors.

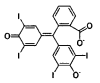
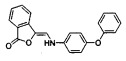
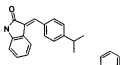
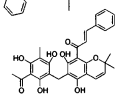
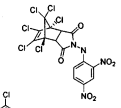
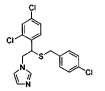
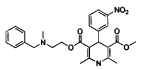
Promiscuous, aggregate-forming inhibitors appear to be relatively common among hits from screening.[17] Their occurrence among true leads, such as quercetin,[18] and drugs, such as sulconazole (Seidler, McGovern, Doman, and Shoichet; *J. Med. Chem.*, in press), suggests that this phenomenon may be common at micromolar concentrations for a broad range of small molecules. The mechanistic studies reported here suggest that inhibition results from the reversible adsorption of enzyme onto the aggregate surface. The ability to prevent or reverse inhibition by the addition of detergent suggests a practical assay for rapid detection of this effect.

Experimental Section

Materials. AmpC β-lactamase and TEM-1 β-lactamase mutants were expressed and purified from *E. coli* as described.[20,21] His-tagged GFP (a gift of Dr. T Uyeda, Advanced Institutes of Science and Technology, Japan) was expressed in *E. coli* with the PET-21b vector and purified under native conditions over Ni-NTA agarose according to the Qiagen QIAexpress protocol. β-Galactosidase, rottlerin, Triton X-100, quercetin, oxalic acid bis(salicylaldehyde hydrazide) (MFCD00187101), 4-(4-bromophenylazo)phenol (MFCD00093929), hexachloro-4-(2,4-dinitrophenylamino)-4-azatricyclo(5.2.1.0(2,6))decenedione (MFCD00225114), tetraiodophenolphthalein (I4PTH), eriochrome blue black (MFCD00003934), moxalactam, sulconazole, and Congo Red were purchased from Sigma-Aldrich (St. Louis, MO). Nitrocefin was purchased from Oxoid (Ogdensburg, NY). Benzo[*b*]-thiophene-2-boronic acid (BZBTH2B) was purchased from Lancaster Synthesis. (4-((2,4-Difluorophenyl)amino)-3,5-thiazolyl)benzene-1,2-diol (MFCD00955233) was purchased from Menai Organics (Gwynedd, N. Wales, U.K.). 3-(4-Isopropylbenzylidene)indolin-2-one (MFCD00118155) was purchased from Maybridge (Tintagel, Cornwall, U.K.). 3-[(4-Phenoxyanilino)methylene]-2-benzofuran-1(3*H*)-one (MFCD00139657) was purchased from Bionet (Camelford, Cornwall, U.K.). Saponin and K-252c were purchased from Calbiochem (San Diego, CA). Nicardipine was purchased from IGN (Pittsburgh, PA). All materials were used as supplied, without further purification.

***β*-Lactamase Assays.** Inhibition assays were performed with AmpC β-lactamase in 50 mM KPi buffer, pH 7.0, at room temperature, as previously described.[18] All assays described here included a 5 min incubation of enzyme and inhibitor, as described.[18] The detergent Triton X-100 was freshly prepared daily as a 1% (v/v) stock in 50 mM KPi. When 0.01% Triton X-100 was included in the incubation (Table 1), it was added before inhibitor or enzyme. When 0.01% Triton X-100 was

Table 3. Effect of 0.01% Triton X-100 on Light Scattering Intensities of Promiscuous, Aggregate-Forming Inhibitors[a]

Compound	IC_{50} vs. β-lactamase (μM)	DLS conc. (μM)	Intensity (kcps)	
			No TX100	0.01% TX100
50 mM KPi	---	---	0.1 ± 0.04	0.1 ± 0.01
	3	10	16 ± 2	0.8 ± 0.3
	8	20	34 ± 2	0.6 ± 0.3
	5	20	25 ± 4	0.3 ± 0.04
	6	20	58 ± 2	0.5 ± 0.1
	1.2	30	14 ± 1	0.6 ± 0.01
	3	15	14 ± 2	1 ± 0.1
	14	40	8 ± 0.5	1 ± 0.1
	20	60	8 ± 0.4	1 ± 0.1

[a] DLS performed in 50 mM KPi at the concentration given under "DLS conc.". kcps is kilocounts per second.

Table 4. Inhibition of β-Lactamase by Promiscuous Inhibitors Dissolved in Potassium Phosphate (KPi) Buffer without DMSO

compd	IC_{50} (μM) vs β-lactamase from DMSO stock	concn in KPi stock (μM)	% β-lactamase inhibition from KPi stock
Congo Red	4	4	81
I4PTH	3	10	72
Quercetin	4	5.9[a]	43
Nicardipine	20	13.5[a]	32

[a] As determined by UV−vis spectra, correlated with solutions diluted from DMSO stocks.

added during the reaction (Figure 5), it was added approximately 100 s after the substrate, 200 μM nitrocefin, was introduced.

Kinetic assays with TEM-1 β-lactamase mutants were performed as described.[20] For assays at 24 °C, inhibitor and enzyme were incubated for 5 min prior to addition of substrate; there was no incubation period for assays at 42 °C. Temperature reflects that of the water bath that fed the water-jacketed cells. The concentration of each mutant was determined spectrophotometrically in concentrated stock solutions. Both mutant enzymes were assayed at 1 nM.

Dynamic Light Scattering. Inhibitors were typically diluted from 10 mM stocks in DMSO with filtered 50 mM KPi. When 0.01% Triton X-100 was present, it was added to the buffer before the inhibitor was added. All compounds were analyzed with a 3 W argon ion laser at 514.4 nm with optical systems from Brookhaven Instrument Corporation. The laser power and integration times were comparable for all experiments. The detector angle was 90°. Each intensity value represents three or more independent measurements at room temperature. For several of the inhibitors described here, DLS measurements were repeated on a DynaPro MS/X, which gave qualitatively similar results.

Cosedimentation Assays. A sample of 3000 ng of AmpC β-lactamase alone, inhibitor alone, or 3000 ng of AmpC β-lactamase and inhibitor was incubated for 5 min at room temperature in 1 mL of 50 mM KPi, pH 7.0. Each solution was centrifuged for 30 min at 13000 rpm (15682*g*) at room temperature, and the supernatant was removed from the pelleted material. The pellet was resuspended with 5 μL of DMSO and 5 μL of 1 mg/mL saponin in 50 mM KPi. To prepare samples for SDS−PAGE, an amount of 10 μL of supernatant or 10 μL of resuspended pellet was mixed with 100 mM DTT and 2% SDS. Each sample was boiled for 5 min at 100 °C. After SDS−PAGE, protein bands were detected by silver staining. Enzyme in the supernatant (Figure 2A, lane 9, and Figure 2B, lanes 7 and 9) was too dilute to detect by our silver-staining method.

Transmission Electron Microscopy. Solutions were prepared to final concentrations of 100 μM I4PTH, 0.1 mg/mL β-galactosidase, with and without 0.0001−1% Triton X-100 in 20 mM Tris-HCl, pH 7.2. At room temperature, 5 μL of each solution was applied to a carbon-coated grid (Electron Microscopy Sciences, Fort Washington, PA) for 30 s. The grid was then blotted on filter paper to remove excess solution, washed three times with 20 mM Tris-HCl, pH 7.2, and negatively stained with 1% aqueous uranyl acetate for 10−20 s. Images were obtained with a JEOL JEM1200 transmission electron microscope at 60 kV. Micrographs were recorded at 15000−60000× magnification.

Confocal Fluorescence Microscopy. A 1.0 mg/mL stock of His-tagged GFP protein was prepared in PBS buffer, a 2 mM stock of I4PTH was prepared in 20 mM Tris-HCl with 2% DMSO, and a 0.03% stock of Triton X-100 was prepared in 20 mM Tris-HCl. Samples of His-GFP alone were prepared by mixing 20 μL of 1 mg/mL His-GFP with 20 μL of PBS buffer and incubating for 2 min, followed by the addition of 20 μL of 20 mM Tris-HCl. Samples containing His-GFP and I4PTH

were prepared by mixing 20 μL of 1 mg/mL His-GFP with 20 μL of PBS buffer and 20 μL of 2 mM I4PTH and incubating for 2 min, followed by the addition of 20 μL of 20 mM Tris-HCl. Samples containing His-GFP, I4PTH, and Triton X-100 were prepared by mixing 20 μL of 1 mg/mL His-GFP with 20 μL of PBS buffer and 20 μL of 2 mM I4PTH, incubating for 2 min, and adding 20 μL of 0.03% Triton X-100. An amount of 100 μL of each sample was placed onto a coverslip (Corning) and sealed with a mixture of Vaseline, beeswax, and lanolin (1:1:1). The GFP fluorescence was visualized using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc., Oberkochen, Germany) equipped with a 100×, 1.4 numerical aperture oil immersion objective. Optical sections were set to ~1.0 μm, and His-tagged GFP was visualized using an excitation at 488 nm and emission at 515−545 nm.

Note Added in Proof. Since submission of this manuscript, a paper had come to our attention and been published by Ryan, Gray, Lowe, and Chung (Effect of detergent on "promiscuous" inhibitors. *J. Med. Chem.* **2003** *46*, 3448−3451) where an effect of detergent on aggregating, promiscuous inhibitors was also described.

Acknowledgment. We thank R. Goldman for generous support of the microscopy experiments, T. Uyeda for providing the GFP construct, J. Seidler for the solubility experiments, P. Stern for introducing us to detergents, J. Horn for introducing us to silver staining, F. Prati for 1-(2-thienylacetylamino)-1-(3-carboxyphenyl)methylboronic acid, the Keck Biophysics Facility of Northwestern University for Brookhaven DLS equipment, and Protein Solutions for partial support of a DynaPro MS/X DLS instrument. We thank K. Olsen, J. Horn, and T. Roth for reading this manuscript. This work was supported by Grant GM59957 from the National Institutes of Health (to B.K.S.). SLM was supported by Grant NIH T32-GM08061 (K. Mayo, PI) and is a Presidential Scholar of Northwestern University.

References

(1) Sun, L.; Tran, N.; Tang, F.; App, H.; Hirth, P.; McMahon, G.; Tang, C. Synthesis and biological evaluations of 3-substituted indolin-2-ones: a novel class of tyrosine kinase inhibitors that exhibit selectivity toward particular receptor tyrosine kinases. *J. Med. Chem.* **1998**, *41*, 2588−2603.

(2) Boehm, H. J.; Boehringer, M.; Bur, D.; Gmuender, H.; Huber, W.; Klaus, W.; Kostrewa, D.; Kuehne, H.; Luebbers, T.; Meunier-Keller, N.; Mueller, F. Novel inhibitors of DNA gyrase: 3D structure based biased needle screening, hit validation by biophysical methods, and 3D guided optimization. A promising alternative to random screening. *J. Med. Chem.* **2000**, *43*, 2664−2674.

(3) Rishton, G. M. Reactive compounds and in vitro false positives in HTS. *Drug Discovery Today* **1997**, *2*, 382−384.

(4) Bajorath, J. Integration of virtual and high-throughput screening. *Nat. Rev. Drug Discovery* **2002**, *1*, 882−894.

(5) Erlanson, D. A.; Lam, J. W.; Wiesmann, C.; Luong, T. N.; Simmons, R. L.; DeLano, W. L.; Choong, I. C.; Burdett, M. T.; Flanagan, W. M.; Lee, D.; Gordon, E. M.; O'Brien, T. In situ assembly of enzyme inhibitors using extended tethering. *Nat. Biotechnol.* **2003**, *21*, 308−314.

(6) Lingameneni, R.; Vysotskaya, T. N.; Duch, D. S.; Hemmings, H. C., Jr. Inhibition of voltage-dependent sodium channels by Ro 31-8220, a "specific" protein kinase C inhibitor. *FEBS Lett.* **2000**, *473*, 265−268.

(7) Soltoff, S. P. Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase Cdelta tyrosine phosphorylation. *J. Biol. Chem.* **2001**, *276*, 37986−37992.

(8) Conseil, G.; Perez-Victoria, J. M.; Jault, J. M.; Gamarro, F.; Goffeau, A.; Hofmann, J.; Di Pietro, A. Protein kinase C effectors bind to multidrug ABC transporters and inhibit their activity. *Biochemistry* **2001**, *40*, 2564−2571.

(9) Davies, S. P.; Reddy, H.; Caivano, M.; Cohen, P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **2000**, *351*, 95−105.

(10) Roche, O.; Schneider, P.; Zuegge, J.; Guba, W.; Kansy, M.; Alanine, A.; Bleicher, K.; Danel, F.; Gutknecht, E. M.; Rogers-Evans, M.; Neidhart, W.; Stalder, H.; Dillon, M.; Sjogren, E.; Fotouhi, N.; Gillespie, P.; Goodnow, R.; Harris, W.; Jones, P.; Taniguchi, M.; Tsujii, S.; von Der Saal, W.; Zimmermann, G.; Schneider, G. Development of a Virtual Screening Method for Identification of "Frequent Hitters" in Compound Libraries. *J. Med. Chem.* **2002**, *45*, 137−142.

(11) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **1997**, *23*, 3−25.

(12) Muegge, I.; Heald, S. L.; Brittelli, D. Simple selection criteria for drug-like chemical matter. *J. Med. Chem.* **2001**, *44*, 1841−1846.

(13) Frimurer, T. M.; Bywater, R.; Naerum, L.; Lauritsen, L. N.; Brunak, S. Improving the odds in discriminating "drug-like" from "non drug-like" compounds. *J. Chem. Inf. Comput. Sci.* **2000**, *40*, 1315−1324.

(14) Xu, J.; Stevenson, J. Drug-like index: a new approach to measure drug-like compounds and their diversity. *J. Chem. Inf. Comput. Sci.* **2000**, *40*, 1177−1187.

(15) Oprea, T. I. Property distribution of drug-related chemical databases. *J. Comput.-Aided Mol. Des.* **2000**, *14*, 251−264.

(16) Walters, W. P.; Namchuk, M. Designing screens: How to make your hits a hit. *Nat. Rev. Drug Discovery* **2003**, *2*, 259−266.

(17) McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K. A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. *J. Med. Chem.* **2002**, *45*, 1712−1722.

(18) McGovern, S. L.; Shoichet, B. K. Kinase inhibitors: Not just for kinases anymore. *J. Med. Chem.* **2003**, *46*, 1478−1483.

(19) Morandi, F.; Caselli, E.; Morandi, S.; Focia, P. J.; Blazquez, J.; Shoichet, B. K.; Prati, F. Nanomolar inhibitors of AmpC β-lactamase. *J. Am. Chem. Soc.* **2003**, *125*, 685−695.

(20) Wang, X.; Minasov, G.; Shoichet, B. K. Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. *J. Mol. Biol.* **2002**, *320*, 85−95.

(21) Weston, G. S.; Blazquez, J.; Baquero, F.; Shoichet, B. K. Structure-based enhancement of boronic acid-based inhibitors of AmpC β-lactamase. *J. Med. Chem.* **1998**, *41*, 4577−4586.

JM030266R