Title
The mechanistic and physical characterization of promiscuous aggregate-based inhibitors

Author
Coan, Kristin Emily deWeese

Publication Date
2008

Peer reviewed|Thesis/dissertation

The Mechanistic and Physical Characterization of Promiscuous Aggregate-Based Inhibitors

by

Kristin Emily deWeese Coan

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry and Chemical Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

I would like to dedicate this dissertation to my parents Martha deWeese and Al Nehl.

Acknowledgements

I have been lucky enough to be surrounded by intelligent, adventurous, and compassionate people who have in many ways made it possible for me to complete this work. I would first like to acknowledge my advisor, Brian Shoichet, for his mentorship, generosity, and patience. I would also like to thank Brian Feng who mentored me on this project, good food and music. For their guidance and support, I would like to thank my thesis committee members, Jim Wells and Sue Miller, and also Pam England, and our program secretary, Chris Olson, who has worked tirelessly to help the students find their ways here at UCSF and to turn in all their forms on time. I would also like to thank my collaborators Joe Goodwin, Dave Maltby, and Al Burlingame.

My parents, Martha deWeese and Al Nehl, have worked very hard to support me in all of my endeavors and I could not have accomplished what I have without their love and encouragement. I would also like to thank my brothers, Nick Coan and Alex Vervloet. In addition, I have been surrounded by many dear friends who have cheerfully accompanied me on many, sometimes harebrained, adventures. Both my lab mates and my classmates have made my experience at UCSF incredibly rewarding and enjoyable. In particular, Kristin Krukenberg, Rafaela Ferreira, Federica Morandi, and Denise Teotico have helped me through difficult times, both scientifically and personally. Last, but certainly not least, Jérôme Hert has been my dearest friend, whose love, support, and sense of humor have enriched my life in countless ways.

This dissertation contains my three first-author publications. The material in Chapter 1 was originally published in *Molecular Biosystems* in 2007. It was reproduced here with permission from K.E.D. Coan and B.K. Shoichet. Stability and equilibria of promiscuous aggregates in high protein milieus. *Molecular Biosystems* **2007,** 3, 208-213. © 2007 *The Royal Society of Chemistry*.

The material in Chapter 2 was originally published in the *Journal of American Society of Chemistry* in 2008. It was reproduced with permission from K.E.D. Coan and B.K. Shoichet. Stoichiometry and physical chemistry of promiscuous aggregate-based inhibitors. *J.A.C.S.* **2008,** 130, 9606-9612. © 2008 *American Chemical Society*.

The material in Chapter 3 has been submitted to the *Journal of Medicinal Chemistry* in 2008. It was reproduced in part with permission from K.E.D. Coan, D.A. Maltby, A.L. Burlingame, and B.K. Shoichet. Promiscuous aggregate-based inhibitors promote enzyme unfolding. *Journal of Medicinal Chemistry*, submitted for publication. Unpublished work copyright 2008 American Chemical Society.

Abstract

The Mechanistic and Physical Characterization of Promiscuous Aggregate-Based Inhibitors

Kristin Emily deWeese Coan

Early drug discovery is plagued by nonspecific molecules, which cannot be developed into drugs. These molecules appear active in initial studies, but upon further investigation are not interesting leads. One of the leading sources of these "false positives" is the formation of large aggregates of organic small molecules, which have the unusual property of promiscuously binding and inhibiting enzymes. Such molecules are common among screening libraries, biological reagents, and even known drugs. Despite their prevalence, there are many elementary properties of these particles that remain unknown and the extent of their repercussions beyond drug screening is not yet clear.

This work focuses on expanding our understanding of the fundamental characteristics and behavior of nonspecific small molecule aggregates. First, to determine whether aggregates may persist in biological contexts we investigated the behavior of these particles in high protein milieus. Second, we explored a number of elementary questions such as the concentration of particles in solution, whether aggregates were in equilibrium with free small molecule monomers, and whether aggregates exhibited micelle-like characteristics such as a "critical aggregation concentration." Finally, we

used hydrogen deuterium exchange mass spectrometry and proteolysis to dissect the molecular principles underlying aggregate-based inhibition.

The key results of this work are as follows. Aggregates are not disrupted by milligram per milliliter concentrations of protein; however, they do exhibit a critical concentration and will rapidly dissociate if diluted below this threshold. Above this threshold, aggregates were detected at femtomolar concentrations. The stoichiometric inhibition of nanomolar enzyme concentrations by femtomolar aggregate concentrations suggests a tight binding interaction between enzyme and aggregate, which is consistent with observations that the off-rate from the aggregate was imperceptible within the timescale of several experiments. Lastly, it appears that aggregates inhibit enzyme by partial unfolding.

Table of Contents

List of Tables

List of Figures

Introduction

One of the dominant methods in early drug discovery is high-throughput screening (HTS).[1, 2] In these screens, drug developers have selected a target protein that they believe is important for treating a disease and whose function can be modulated by a small molecule drug.[3] By searching through hundreds of thousands of small molecules they aim to find molecules that will interact productively with their selected target. To test so many molecules the target protein is isolated from its complex, native environment and placed into a simplified, *in vitro* system. In these simplified conditions it is possible to rapidly test vast numbers of potentially useful small molecules.

There have been successes with HTS, but this technique also has some notable weaknesses.[4-6] First, the rarified conditions of *in vitro* assays de-emphasize the behavior of a small molecule *in vivo*, which is ultimately the most important consideration. How the molecule will be transformed in different biological environments, how it will reach its target, and whether the molecule will affect other enzymes and produce side effects are just a few of many crucial considerations. The importance of these considerations has been suggested to contribute to the disappointing number of new drugs that have been discovered since the advent of HTS and other advances in drug discovery.[7-10]

Another shortcoming of HTS is its sensitivity to false positives – molecules that appear to have desired activity in the screen, but upon further study are nonspecific or artifactual. Hit rates are typically only a small percentage of the screened library, and the majority of these "hits" are usually false positives.[11, 12] Sorting the false from the genuine

leads requires many additional assays and a great deal of time and money can be spent before it becomes clear that the molecules are unsuitable drug candidates.

False positives are attributed to several causes. Sometimes, a molecule can simply interfere with the assay (this is particularly common with fluorescence-based assays).[13] Other common causes include genuinely promiscuous molecular scaffolds or reactive molecules; however, these mechanisms do not account for a large portion of false positives.[12, 14, 15] Additionally, many false positives show unusual characteristics such as steep dose-response curves, nonexistent structure activity relationships, and poor specificity. Such molecules are common among screening hits, but until relatively recently the explanation for their apparent inhibition was unknown.

In 2002, McGovern *et al*. discovered that there was a unifying mechanism that explained a large number of these false positives.[16] They found that a number of drug-like small molecules formed large, submicron aggregates in solution. These aggregates had the peculiar property of nonspecifically binding and inhibiting enzymes. The particles formed at micromolar concentrations of the small molecule, in the same range as many drug companies use in their screens and these particles appeared completely promiscuous – they inhibited every enzyme tested. Feng *et al*. developed a high-throughput screen for the early detection of aggregating molecules and subsequently found that this mechanism may represent the most common cause of false positives.[17] In the following years, the news was even more worrisome: these molecules were not limited to screening libraries. Biological reagents like kinase and amyloid inhibitors and even marketed drugs exhibited aggregate behavior *in vitro*.[18-20]

For such a widespread problem, very little was known about the aggregates themselves. A number of questions remained about their implications in drug discovery and basic research, but the aggregates themselves merited further study as well. Further characterization may aid in understanding their behavior in varied contexts, but aggregates also represent a new mode of interaction between small molecules and enzymes. These molecules are affecting biological systems by an unusual mechanism that we have yet to fully understand.

Given that drugs can behave as aggregates *in vitro*, one of the more pressing issues was to determine how they might behave *in vivo*. Other groups suggested that these large aggregates may persist in the gut and that this would have implications for drug distribution and action. Previous research from our group had shown that serum albumin can prevent inhibition by small molecule aggregates, but the mechanism was unknown. One common hypothesis was that high concentrations of protein may disrupt the aggregates, thus preventing their formation *in vivo*. To investigate whether aggregates might form in biological milieus, we studied their behavior at high concentrations of protein, as would be found in a biological environment. We measured the aggregate formation of four known aggregating molecules in the presence of three different proteins and found that high concentrations of protein were insufficient to prevent or disrupt aggregate formation. This work is discussed in Chapter 1.

Despite their prevalence, there were fundamental characteristics about aggregation that were still unknown. For example, we could not measure the concentration of the particles, although we knew the concentration of the small molecule. From the concentration, it would be possible to investigate other characteristics such as

the structure of the aggregates (are they hollow or solid?), the stoichiometry of the enzyme-aggregate interaction, and whether enzyme is absorbed within the aggregate or adsorbed to the surface. To measure the concentration of aggregate particles, we used a flow cytometer specifically optimized to look at aggregation. We counted the number of particles formed by seven known aggregators and found that the concentration of particles near the threshold of aggregate formation was typically in the femtomolar range. The complete characterization of two of these aggregates is described in Chapter 2.

Finally, we wanted to further investigate the specific mechanism of aggregate-based inhibition by determining the structural changes that occur when enzyme is bound to an aggregate. There was evidence suggesting that aggregates did not denature enzyme, so we hypothesized that aggregates may be inhibiting enzyme by partially unfolding the enzyme, reducing dynamic motions, or physically sequestering the enzyme. To determine which of these potential mechanisms may be true, we investigated changes in solvent accessibility that occurred when β-lactamase was bound by the aggregator rottlerin. We also assayed changes in protease susceptibility in the presence of five aggregating molecules. Our results suggest that partial unfolding is the most likely mechanism of action for these aggregates. This work is presented in Chapter 3.

Gloss to Chapter 1.

Small molecule aggregation is a widespread problem with particular relevance to high-throughput screening, a dominant method in early drug discovery. Here, "hits" due to nonspecific inhibition by aggregation may represent the leading cause of false positives. An additional troubling possibility is that the problem may not be limited to *in vitro* screens. Known drugs and biological reagents can behave as promiscuous aggregates *in vitro* and this naturally leads one to wonder if these molecules can also aggregate in the biological contexts where they are applied.

Frenkel *et al*. discovered a handful of potentially therapeutic non-nucleoside reverse transcriptase inhibitors that aggregated at relevant concentrations.[21] They went on to propose that aggregation in the gut was important to the distribution of their molecules *in vivo*. Clearly, if drugs were aggregating *in vivo* this may have broad impacts on drug action and distribution. One implication might be that drugs are being sequestered in large colloid-like aggregates, decreasing the effective concentration throughout the body. To investigate the potential behavior of aggregates in such a context, we used a simplified system that mimicked one aspect of biological environments: high protein concentrations.

In the early discovery of aggregators, McGovern *et al*. noted that inhibition could be prevented by the addition of serum albumin.[16] The mechanism at that point was unknown, but many suspected that high concentrations of protein may disrupt small molecule aggregates. To determine whether this was true, we studied aggregation of four aggregating molecules in the presence of milligram per milliliter concentrations of three

unrelated enzymes or proteins, including serum albumin. We continued to observe particle formation by dynamic light scattering regardless of the presence of protein. This indicated that high concentrations of protein could not prevent the formation of aggregates or disrupt them once formed.

So, why did serum albumin prevent inhibition? Our results suggested that serum albumin, and other proteins, could not disrupt aggregates. At this point, it is key to note that McGovern determined whether inhibition could be *prevented*, meaning that the aggregates and serum albumin were combined prior to the addition of β-lactamase – the monitored enzyme. This led us to hypothesize that the addition of an excess amount of protein simply saturates the aggregate, preventing it from binding subsequently added enzyme. This is also consistent with the unusual sensitivity of aggregate-based inhibition to enzyme concentration: a ten-fold increase in β-lactamase concentration causes enzyme activity to return.

Another question was whether inhibition could be *reversed* by the addition of an excess amount of protein. One might imagine that there is a measurable equilibrium between aggregate-bound and free enzyme. If there is a great excess of another protein in solution, such as serum albumin, then the bound enzyme, say β-lactamase, could eventually be replaced by the excess serum albumin. In other words, whenever β-lactamase samples the unbound state, it is far more likely that a molecule of serum albumin will find its way to the now empty binding site on the aggregate rather than the β-lactamase molecule. If this were true, we would expect β-lactamase activity to return as the bound β-lactamase is displaced by serum albumin.

To determine whether inhibition could be *reversed* by excess protein, we assayed β-lactamase inhibition by four aggregates in the presence of three additional enzymes. β-lactamase was incubated with the aggregates for five minutes prior to the addition of the competing protein. For three of the compounds we saw no reversal, suggesting that the equilibrium strongly favored the bound state and that the off rate from the aggregate was not measurable within the timescale of our experiments. Furthermore, we found that if we combined aggregate-bound enzyme with a specific irreversible inhibitor, the enzyme was protected by the aggregate from the second inhibitor. This indicated (a) that the enzyme spent no significant time free in solution and (b) that aggregate-bound enzyme was sequestered in such a way that it could not even perform the covalent half reaction necessary to bind the irreversible inhibitor.

There was one exception to these results. We found that serum albumin had some limited ability to disrupt aggregates of the small molecule tetraiodophenolphthalein. In enzyme assays we also saw modest reversal of inhibition when serum albumin was added in milligram per milliliter quantities. One of the functions of serum albumin in the blood is to absorb a variety of small molecules and here we supposed that we were observing specific binding of the small molecule by serum albumin.[22] The aggregates were disrupted because albumin was binding free small molecule and shifting the equilibrium away from the aggregate form.

The clear results from this work were that high concentrations of protein are typically not sufficient to disrupt small molecule aggregates and that the binding interaction between enzyme and aggregate appears to be remarkably tight. Although we cannot make any definitive statement about aggregate behavior *in vivo*, our results

indicate that the subject merits further exploration. The paper describing this work was published by *Molecular Biosystems* in the spring of 2007.

Chapter 1.

1. Stability and equilibria of promiscuous aggregates in high protein milieus

*Kristin E.D. Coan and Brian K. Shoichet**

Department of Pharmaceutical Chemistry & Graduate Group in Chemistry and Chemical Biology

1700 4th St., University of California at San Francisco, San Francisco, California 94158-2550

Abstract

At micromolar concentrations, many molecules form aggregates in aqueous solution. In this form, they inhibit enzymes non-specifically leading to false positive "hits" in enzyme assays, especially when screened in high-throughput. This inhibition can be attenuated by bovine serum albumin (BSA); the mechanism of this effect is not understood. Here we present evidence that BSA, lysozyme, and trypsin prevent inhibition when incubated at milligram per milliliter concentrations with aggregates prior to the addition of the monitored enzyme. These solutions still contained aggregates by dynamic light scattering (DLS), suggesting that inhibition is prevented by saturating the aggregate, rather than disrupting it. For most combinations of aggregate and protein, inhibition was not reversed if the competing protein was added after the incubation of aggregates with the monitored enzyme. In the one exception where modest reversal was observed, DLS and flow cytometry indicated that the effect was due to the disruption of aggregates. These results suggest that aggregate-bound enzyme is not in dynamic equilibrium with free enzyme and that bound enzyme cannot be displaced by a competing protein. To further test this hypothesis, we incubated aggregate-bound enzyme with a specific, irreversible inhibitor and then disrupted the aggregates with detergent. Most enzyme activity was restored on aggregate disruption, indicating no modification by the irreversible inhibitor. These results suggest that enzyme is bound to aggregate so tightly as to prevent any noticeable dissociation and that furthermore, aggregates are stable at physiologically relevant concentrations of protein.

Introduction

Many organic molecules form colloid-like aggregates at micromolar concentrations in aqueous solution.[16, 23] These micron-sized particles inhibit enzymes promiscuously, contributing significantly to false positive "hits" in high-throughput screening; the role of these aggregates as artifacts in biochemical assays is now widely accepted.[12, 16, 24-27] Promiscuous aggregators have also been found among commonly used biological reagents, such as kinase inhibitors, Lipinski-compliant small molecules, and drugs.[19, 20, 28-33] Despite their prevalence, relatively little is understood about the mechanism of inhibition and even less about the behavior of such aggregates in a biological context.[34, 35] Recently, however, Frenkel et al. proposed that small molecule aggregation can occur *in vivo* and play a role in the bioavailability of certain drugs in the body.[21] These investigators suggested that aggregates of non-nucleoside reverse transcriptase inhibitors form and persist in the gastrointestinal tract on oral dosing. The aggregates would then be absorbed by particle-recognizing M cells in Peyer's patches of mucosa-associated lymphoid tissue, ultimately resulting in lymphatic distribution. To revisit the behavior of aggregates in a biological milieu, we wanted to consider the effect of high protein concentrations, as might be found *in vivo*, on these particles.

In the initial studies of aggregates, McGovern et al. found that inhibition could be prevented by the addition of milligram per milliliter concentrations of BSA prior to the addition of the aggregating inhibitor and monitored enzyme.[16] Why this should be true is unclear and we considered two possible explanations for this effect: either the addition of BSA simply pre-saturates the aggregates or BSA actually prevents the formation of

aggregates. In addition, is this property unique to BSA, which is known for its ability to bind small molecules,[22, 36] or would the presence of any protein have a similar effect? If aggregates do not form, or if they are disrupted in the presence of BSA, this would suggest that aggregates might not survive a protein-rich physiological environment. Conversely, if aggregates are still present, supporting the pre-saturation model, the potential role of aggregates in drug bioavailability may merit further exploration. Mechanistically, we also wondered whether inhibition could be *reversed* by adding protein after the formation of enzyme-aggregate complexes. Reversibility would suggest a dynamic equilibrium between free and aggregate-bound enzyme and reflect on the stability of the enzyme-aggregate complex.

Here we investigate these questions by monitoring the inhibition of a β-lactamase when competing proteins are added before or after incubation with aggregates. These experiments address whether attenuation of inhibition is specific to BSA, whether attenuation results from saturation or disruption of aggregates, and whether it is possible to reverse inhibition with any protein. To do so, we use three unrelated competitor proteins, four aggregators, and a chemically reactive, irreversible β-lactamase inhibitor that probes the accessibility of the β-lactamase active site. These results provide insight into the dynamic equilibria of enzyme-aggregate complexes and the stability of these complexes at the high protein concentrations they experience in biological environments. This work suggests that protein ameliorates the effects of aggregating inhibitors, but that the aggregate and the aggregate-enzyme complex may be quite persistent.

Experimental

Materials. AmpC β-lactamase was expressed and purified as described.[37] Chicken egg white lysozyme, porcine pancreatic trypsin, tetraiodophenolphthalein (TIPT), 4-(4-bromophenylazo)phenol (4BPAP), S3218, rottlerin, Triton X-100 and moxalactam were purchased from Sigma-Aldrich. Nitrocefin was purchased from Oxoid. Centa was a gift from M. Paola Costi, University of Modena. All materials were used as supplied by the manufacturer.

β-lactamase Assays. β-lactamase activity and inhibition was monitored in 50 mM potassium phosphate (KPi) buffer, pH 7.0, at room temperature. Nitrocefin was prepared as a 20 mM stock in dimethyl sulfoxide (DMSO) and DMSO stocks of aggregating inhibitors were prepared so that the total concentration of DMSO was less than 2 %. Results were controlled for the effect of DMSO on enzyme rates. Inhibitor and 1 nM β-lactamase were incubated for five minutes and the reaction was initiated by the addition of 200 μM nitrocefin substrate. Change in absorbance was monitored at 482 nm for 100 seconds. To determine preventative effects, 0.1 mg/mL BSA, lysozyme, or trypsin was added to the buffer before the addition of inhibitor or β-lactamase. For reversal experiments, 1 mg/mL BSA, lysozyme, or trypsin was added after a five minute incubation of β-lactamase with the inhibitor and the reaction was initiated by substrate immediately thereafter. In the case of TIPT, 0.1 and 10 mg/mL BSA were also tested.

The covalent inhibitor, moxalactam, was prepared as a 0.2 mM stock in 50 mM KPi. The substrate centa was prepared as 12.5 mM stock in 50 mM KPi buffer. The detergent Triton X-100 was freshly prepared daily as a 2 % (v/v) stock in 50 mM KPi.

For reactions with centa, 125 μM of this substrate was used and absorbance at 405 nm was monitored. When investigating covalent inhibition by moxalactam, TIPT and β-lactamase were incubated for five minutes before the addition of moxalactam, which was then incubated another five minutes before the reaction was initiated. Approximately 100 seconds after the addition of centa, 0.04 % Triton X-100 was added to disrupt aggregates.[34]

Dynamic Light Scattering. Inhibitors were delivered from concentrated DMSO stocks and diluted with filtered 50 mM KPi buffer, pH 7.0. Preventative effects were measured by including 0.1 mg/mL BSA, lysozyme, or trypsin in the buffer before the addition of the inhibitor. The effect of 0.1 mg/mL BSA on aggregate disruption was determined by the addition of a small volume of concentrated BSA to a solution of pre-formed aggregates. To measure disruption in the presence of β-lactamase, aggregates were incubated first with 1 nM β-lactamase, and then BSA was added. Measurements were made using a DynaPro MS/X with a 55 mW laser at 826.6 nm. The laser power was 100 % unless noted and the integration time was 200 seconds. The detector angle was 90°. Each intensity value represents twenty independent measurements at room temperature.

Flow Cytometry. Particle characterization was performed using a BD GentestTM Solubility Scanner, a flow cytometer adapted to detect colloids and particles by light scattering. Mixtures were made in a 96-well plate with a final volume of 200 μL per well. TIPT was diluted into filtered 50 mM KPi from a 5 mM stock in DMSO. The final concentration of DMSO was 1 % and the results were controlled for effect of DMSO. BSA was delivered from a 10 mg/mL stock in 50 mM KPi buffer, pH 7.0. Measurements were acquired with a 3 mW laser at 635 nm. Photon signatures were collected at 90° with

a PMT setting of 100. The threshold channel was set to 25 with a flow rate of 0.5 μL per second.

Results

To explore the mechanism of BSA-dependent attenuation of promiscuous inhibition, we determined first whether this characteristic was unique to BSA or whether the addition of other proteins would have a similar effect. We chose two unrelated enzymes, trypsin and lysozyme, to test in addition to BSA. Four aggregates were tested (TIPT, 4BPAP, S3218, and rottlerin, Fig 1) at a concentration between two and four-fold their IC_{50} values. In the absence of competing protein, β-lactamase activity was consistently inhibited at least eighty percent by the aggregates. Consistent with prior studies[16], when 0.1 mg/mL BSA, lysozyme, or trypsin was added to the solution before β-lactamase, no significant inhibition was observed (Table 1). The one exception to this was TIPT combined with lysozyme and trypsin, where some residual inhibition persisted. Even here, inhibition was greatly attenuated, especially considering that the concentration of TIPT was four-fold above the IC_{50}.

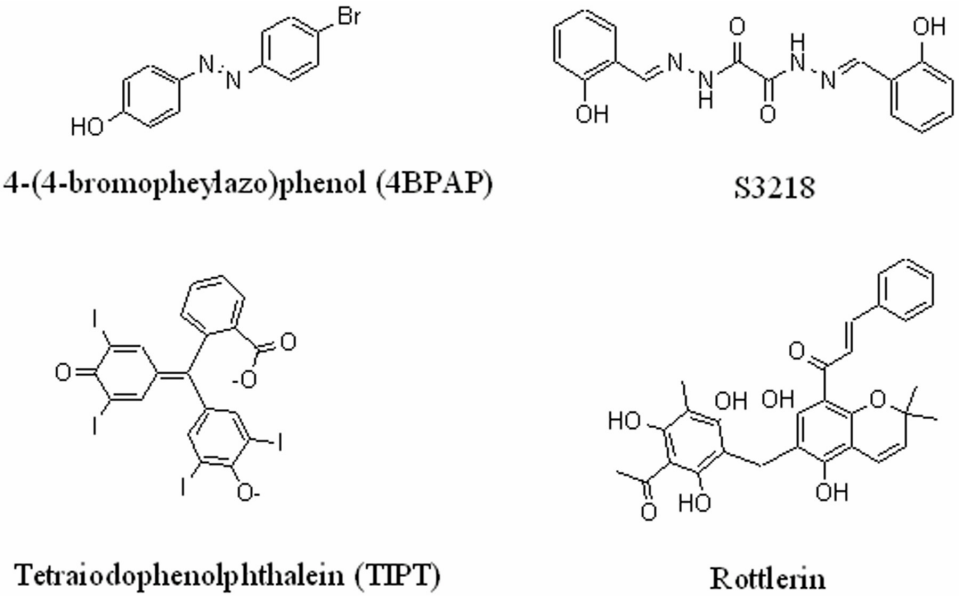


Figure 1. Aggregators used in this study.

Table 1. β-lactamase inhibition and aggregate formation after pre-incubation with BSA, lysozyme, and trypsin.

Aggregate	Conc. (μM)	IC_{50}	% β-lactamase Activity		DLS Intensity (kcps)		
			No Protein	0.1 mg/mL Protein	No Protein	0.1 mg/mL Protein	Protein only
Protein: Bovine Serum Albumin							
TIPT	20	5	8	91	5952	3148	94
S3218[a]	30	17	12	101	2910	3710	14
4BPAP	20	9	2	90	1749	2360	191
rottlerin	5	1.5	14	89	N.M.[b]	N.M.	N.M.
Protein: Lysozyme							
TIPT	20	5	7	75	5952	5677	225
S3218[a]	30	17	5	102	2910	4663	115
4BPAP	20	9	2	99	1749	2130	35
rottlerin	5	1.5	13	97	N.M.	N.M.	N.M.
Protein: Trypsin							
TIPT	20	5	6	38	5952	5660	33
S3218[a]	30	17	5	98	2910	4636	7
4BPAP	20	9	2	96	1749	2042	149
rottlerin	5	1.5	16	86	N.M.	N.M.	N.M.

[a] S3218 light scattering was measured at 50% laser power.

[b] Not measured. Rottlerin does not form particles by DLS below 10μM.

Since the protecting protein was present in the solution before the addition of the aggregating small molecule, it could act by either (a) preventing aggregate formation or (b) saturating the aggregate so that it was unable to bind β-lactamase. In the first model, we would expect significantly fewer particles in solution, whereas if the number of aggregates was relatively unchanged, it would support the second model. To determine whether or not aggregates were forming, solutions containing protein and aggregate were analyzed by dynamic light scattering. Every solution containing protein with aggregate scattered a comparable amount of light to their aggregate-only counterparts, suggesting an equivalent population of aggregates both in the absence and presence of protein (Table 1). None of the proteins alone in buffer scattered significantly.

To determine whether it was possible to restore activity by the addition of protein *after* the formation of an aggregate-enzyme complex, we performed enzyme inhibition assays in the presence of varied competing proteins. Each protein was added subsequent to the incubation of β-lactamase with aggregate. Dose-response curves were obtained for TIPT, S3218, and rottlerin in the presence of 1 mg/mL of BSA, lysozyme, and trypsin (Fig 2). Rottlerin and S3218 showed no sensitivity to any of the three proteins. TIPT inhibition was slightly reversed upon the addition of BSA, but not the other two proteins. We then obtained dose-response curves for TIPT in the presence of no protein, 0.1 mg/mL, 1 mg/mL, and 10 mg/mL BSA (Fig 3). Although some activity was restored by BSA, this effect was modest: at 10 mg/mL BSA, the IC_{50} of TIPT barely doubled from 5 to 10 μM.

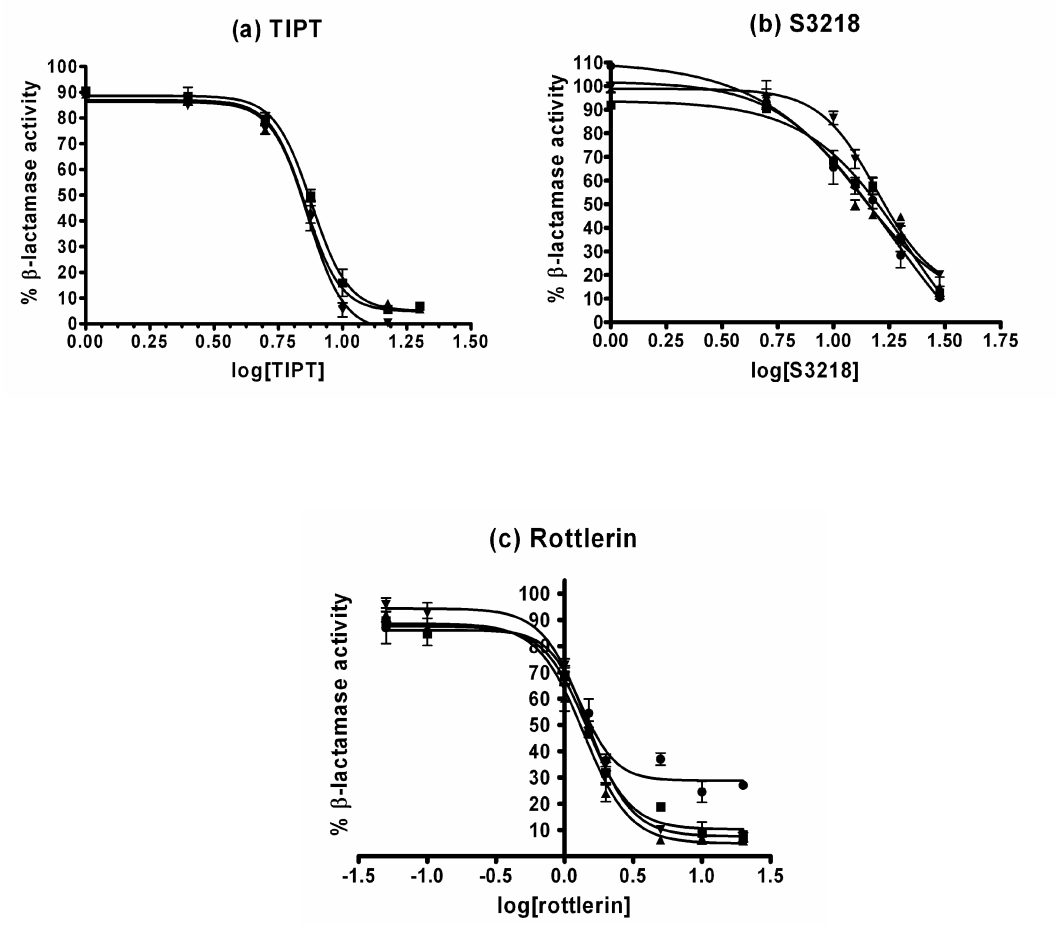


Figure 2. β-lactamase inhibition by varied aggregators after the addition of competing proteins. β-lactamase inhibition by (a) TIPT, (b) S3218, and (c) rottlerin after the addition of competing proteins. Competing proteins were added after incubation of aggregate and β-lactamase: no protein (■), 1 mg/mL BSA (●), 1 mg/mL lysozyme (▲), and 1 mg/mL trypsin (▼). Dose-response curves for TIPT in the presence of BSA are shown in Fig 3. Error bars represent the standard error of the mean for three replicate measurements.

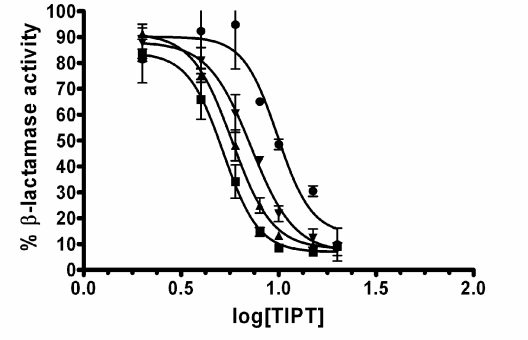


Figure 3. BSA-dependent reversal of β-lactamase inhibition. BSA-dependent reversal of β-lactamase inhibition by TIPT with no protein (■), 0.1 mg/mL BSA (▲), 1 mg/mL BSA (▼), and 10 mg/mL BSA (●). TIPT and β-lactamase were incubated together for five minutes prior to the addition of BSA. Error bars represent the standard error of the mean for three replicates.

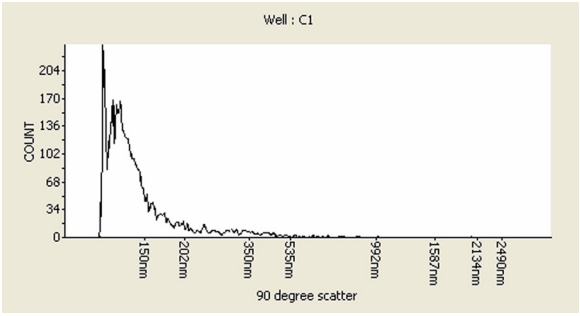
Although reversal was only observed for the specific case of BSA and TIPT, we investigated the mechanism using dynamic light scattering and flow cytometry. Reversal could result either from disruption of aggregates or from displacement of aggregate-bound enzyme by the additional protein. The second model would indicate equilibration between free and aggregate-bound enzyme with at least a transient population of free enzyme. The reduction or maintenance of particles would support each hypothesis, respectively. Addition of 0.1 mg/mL BSA to TIPT in buffer led to a large drop in dynamically scattered light, consistent with aggregate disruption (Table 2). This reduction became attenuated at the highest concentration of TIPT, suggesting that aggregates reappeared by 20 μM TIPT (consistent with the presence of aggregates after pre-incubation with BSA in Table 1). Light scattering was also monitored in the presence of 1 nM β-lactamase, matching the conditions of the enzyme assay. Disruption by BSA was less pronounced in these solutions, but still occurred. We followed the DLS studies with flow cytometry, where aggregates are flowed in a narrow (0.5 μm) stream across a laser field and a scattering detector. In the absence of BSA, the size distribution of TIPT is centered at approximately 100 nm (Fig 4). Upon the addition of 0.1 mg/mL BSA to 10 μM TIPT in buffer, this population disappeared and no other particles were detected. Both results support the disruption of TIPT aggregates by BSA, but only within a limited concentration range.

Table 2. BSA-dependent disruption of TIPT aggregates at varied concentrations.

[TIPT] μM	DLS Intensity (kcps)			
	No β-lactamase		+ 1 nM β-lactamase[a]	
	No BSA	+ 0.1 mg/mL BSA[b]	No BSA	+ 0.1 mg/mL BSA
10	774	110	2502	703
15	4101	291	5661	2717
20	6166	2709	N.M.[c]	N.M.

[a] β-lactamase only scatters 10 kcps.
[b] BSA alone scatters 31 kcps.
[c] Not measured.

(a)



(b)

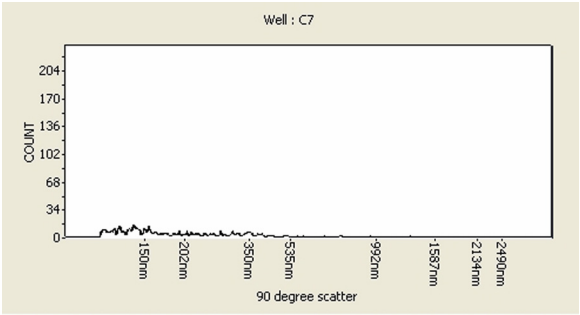


Figure 4. Size distribution histograms obtained by flow cytometry. Size distribution histograms obtained by flow cytometry for (a) 10 μM TIPT and (b) 10 μM TIPT + 0.1 mg/mL BSA.

To further explore whether there was any significant equilibrium between free and aggregate-bound enzyme, we assayed β-lactamase inhibition by moxalactam, a reactive, irreversible inhibitor, in the presence of aggregates. We reasoned that there were two possible mechanisms for how moxalactam might irreversibly inhibit aggregate-bound enzyme: (1) bound enzyme retains enough activity to undergo a covalent modification to the active site or (2) aggregate-bound enzyme is in dynamic equilibrium with free enzyme (Fig 5). Alternatively, if aggregate-bound β-lactamase is not irreversibly inhibited by moxalactam, it refutes both mechanisms. This would suggest that there is no measurable dynamic equilibrium between bound and free enzyme and that the enzyme active site is protected from covalent modification. β-lactamase was incubated initially with TIPT, and subsequently with moxalactam. Upon the addition of the non-ionic detergent Triton X-100, which disrupts aggregates and releases active enzyme,[34, 38] β-lactamase activity was restored. The initial amount of enzyme activity restored in the presence of TIPT and moxalactam was no less that that restored by β-lactamase that had been incubated with TIPT alone (Fig 6). As expected, time-dependent inhibition of the free enzyme by moxalactam became apparent approximately 100 seconds after aggregate disruption. β-lactamase that had been incubated with moxalactam alone, without aggregates, showed complete inhibition and no reversal upon the addition of detergent (data not shown). Since the presence of the aggregate resulted in complete protection of β-lactamase from moxalactam, we concluded that there was no measurable dynamic equilibrium between aggregate-bound and free enzyme. This is consistent with a tight effective K_d between aggregate and enzyme, which is supported by other recent studies.[39]

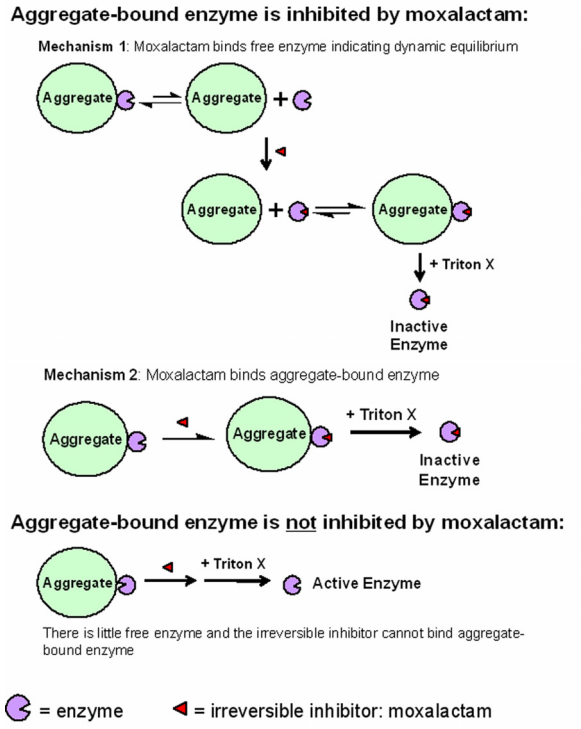


Figure 5. Proposed interpretations for the accessibility of an enzyme to an irreversible inhibitor in the presence of aggregates. Enzyme-aggregate complexes are incubated with an irreversible inhibitor prior to the disruption of aggregates by the detergent Triton X-100. If aggregate-bound enzyme is not inhibited by moxalactam, both Mechanism 1 and 2 are refuted. Aggregates are shown in green, enzyme in purple, and the irreversible inhibitor is represented by a red triangle (see key at bottom of figure).

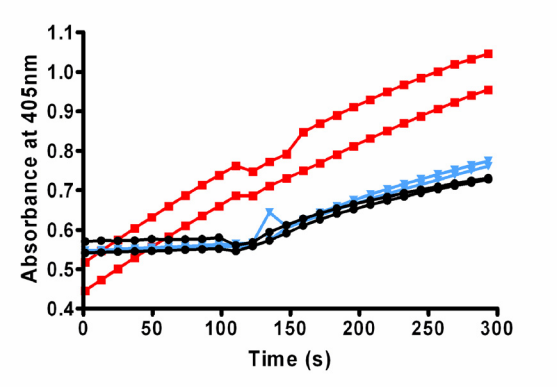


Figure 6. Aggregate disruption by Triton X-100 in the presence of an irreversible inhibitor. Aggregate disruption by 0.04% Triton X-100 added during a β-lactamase inhibition assay containing β-lactamase (red, ■), β-lactamase with 25 μM TIPT (blue, ▼), and β-lactamase treated first with 25 μM TIPT followed by incubation with 1 μM moxalactam (black, ●). Triton X-100 was added approximately 100 seconds after the reaction was initiated with substrate.

Discussion

It is tempting to believe that the high protein concentrations of biological milieus would be sufficient to disrupt colloidal aggregates of small molecules, which are so pernicious in biochemical assays.[23, 25, 26, 32] These studies do not support that view. Although aggregates can be saturated by protein, they are not typically disrupted at milligram per milliliter concentrations of protein. Furthermore, once formed, the aggregate-enzyme complex is unperturbed by additional protein. Thus, whereas the inhibitory effect of aggregates is mitigated, even eliminated, by pre-exposure to additional protein, the aggregate species itself persists, potentially viable in a more biological environment. From a mechanistic standpoint, the inability to compete off a pre-associated protein with a second, additional protein has implications for our understanding of the dynamic equilibrium to which protein-aggregate complexes are subject.

One question we had from the outset was whether BSA attenuation of aggregate-based inhibition was a specific property of that protein or whether attenuation could be achieved by any protein. One might imagine that BSA is unique, given the role of serum albumin as a carrier and reservoir of physiological organic molecules.[22, 36] However, our results indicate that three proteins – BSA, lysozyme, and trypsin – all prevented inhibition by four aggregates at concentrations between two and four-fold their IC_{50}s (Table 1). Having demonstrated that attenuation was not specific to BSA, we wanted to determine the mechanism of this attenuation. We reasoned that a high concentration of protein present in the solution before the addition of the aggregating molecules might

have some impact on aggregate formation. Alternatively, aggregates may form as usual, but the protein present would saturate the aggregate, precluding any binding of the subsequently added β-lactamase. Consistent with a pre-saturation model, dynamic light scattering by aggregates was typically unaffected by the presence of high protein concentrations.

Contrary to the profound preventative effects, additional protein generally showed no ability to *reverse* inhibition. We had suspected that a large excess of competing protein might displace aggregate-bound enzyme, releasing enzyme. Since we know from previous work that disruption of aggregate-enzyme associations with detergent restores activity to the enzyme, we would also expect dissociated enzyme to be active.[34] To our surprise, inhibition by three aggregates was unaffected by the addition of up to a 1000-fold excess of competing protein (Fig 2). This suggested that there was no measurable equilibrium between free and aggregate-bound protein: once the enzyme-aggregate complex was formed it was imperturbable, at least within the time scale of our experiments. Preliminary experiments indicated that longer incubation times did not significantly restore activity (data not shown). There was only one exception to this trend: the specific combination of TIPT and BSA. Here, BSA actually *reversed* TIPT inhibition (Fig 3). Even so, this reversal appeared to be the result of disruption, rather than an equilibrium. Dynamic light scattering and flow cytometry indicated that BSA disrupted TIPT aggregates, but only at lower concentrations of the aggregating molecule (Table 2, Fig 4). This reversal was ultimately modest: even at 10 mg/mL BSA, TIPT IC_{50} values barely doubled and by 20 μM TIPT, particles and inhibition had returned.

We further probed the existence of a dynamic equilibrium between free and aggregate-bound enzyme using an irreversible inhibitor of β-lactamase. If there is no measurable equilibrium, we would expect aggregate-bound enzyme to be unaffected by incubation with such an inhibitor. Consistent with this view, aggregate-bound β-lactamase is inaccessible to the irreversible inhibitor moxalactam, even after a 30 minute incubation (data not shown), suggesting that β-lactamase spends no significant time free in solution. These results suggest that the effective K_d between enzyme and aggregate is low enough as to make dissociation immeasurable on the time-scale of these experiments, a view that is consistent with recent studies of the limiting K_d value of enzyme-aggregate complexes.[39] Furthermore, since enzyme is protected from covalent modification while bound to the aggregate, this also implies that in addition to being catalytically inactive, the enzyme cannot even bind a substrate-like molecule.

Conclusions

Given the widespread presence of aggregating molecules, a comforting thought has been that aggregation may be restricted to the elementary conditions of biochemical assays. Work by other groups has suggested, however, that colloidal aggregates of small molecules may be stable in biological, even whole animal milieus.[21] Our study, which explores one aspect of biological environments – a high protein concentration – is compatible with that proposal. Although inhibition can be prevented by milligram per milliliter concentrations of protein, it is rarely reversed. The potential activity of these aggregates in a physiological context remains an open area of research, but this study suggests that aggregates are at least stable, and not disrupted, in high protein

environments. Since many molecules, including drugs and reagents, aggregate at micromolar concentrations, the possible fates of aggregates in biological systems may merit further study.

Acknowledgements

This work was supported by NIH grant GM71630 (to BKS). KEC was partly supported by NIH grant T32-GM64337 (C. Craik, PI). We thank J. Goodwin and BD Biosciences for the generous loan of the BD Gentest™ Solubility Scanner and for helpful discussions and F. Morandi for β-lactamase purification. We also thank B. Feng, K. Babaoglu, R. Ferreira and V. Thomas for reading this manuscript.

Gloss to Chapter 2.

As of 2007, nearly five years after the discovery of aggregates and after nearly a dozen papers had been published on the subject, there were still fundamental questions that remained unanswered. For example, we had no way of measuring something as elementary as the concentration of the aggregate particles. Determining this concentration would allow us to address a number of other long-standing questions including whether aggregates are hollow or solid, the stoichiometry of the aggregate-enzyme interaction, and whether aggregates exhibit a "critical aggregation concentration."

Although the concentration of small molecule in solution was known, it was difficult to measure the concentration of the particles. Aggregates are too small to be detected by the techniques typically used to count cells, such as microscopy and flow cytometry. Feasible methods for counting aggregates did not exist until Joe Goodwin and colleagues at BD Bioscience optimized a flow cytometer specifically to analyze small molecule aggregates. Once we had this instrument, we were able to learn a great deal about the basic behavior and characteristics of aggregates.

First, we measured the aggregate concentration for seven aggregators and found that near the threshold of aggregation formation the concentration was often low femtomolar. As we had suspected aggregates resemble micelles in that they exhibit a critical concentration where aggregates begin to form. Above this concentration, all additional small molecules form aggregates and the concentration of free small molecule remains constant, while the aggregate concentration increases linearly. Interestingly, this process worked in reverse as well. When aggregates were diluted below this "critical

aggregation concentration," they dissociated and returned to their free small molecule components within minutes. This marks aggregates as unique from precipitant, where redissolving material often requires hours to days.

The flow cytometer also provided size distributions for all samples, so we monitored the size of the particles as well as their concentration. Previous DLS experiments suggested that aggregates have distinct size preferences. This became more apparent by flow cytometry. Some aggregators formed larger particles around 500 nm in diameter, such as nicardipine and miconazole, whereas many more molecules preferentially formed smaller particles around 100 nm. The aggregates maintained this size preference across varied concentrations. At the writing of this paper, 100 nm in diameter was near the limits of the flow cytometer and so we were unable to analyze these aggregates in greater detail, but we were able to get reliable data for the two larger aggregates: nicardipine and miconazole.

From the aggregate concentration, we were able to correlate β-lactamase inhibition to particle counts and so estimate the stoichiometry of the enzyme-aggregate interaction. We found that each of these aggregates bound on the order of 10,000 enzyme molecules per aggregate particle. From this calculation, we also wanted to estimate whether this quantity of enzyme could fit comfortably on the surface of the aggregate, to explore whether aggregates adsorb or absorb enzyme. Although we still could not rule out absorption as a mechanism, this analysis at least gave us an indication of whether the surface is sufficient to accommodate all bound enzyme. By calculating the volume of a thin shell around the aggregate (the width of the shell being the smallest dimension of a

β-lactamase molecule), we found that the surface provided more than enough binding space for even 10,000 β-lactamase molecules.

Finally, we addressed one last question: are aggregates hollow or solid? By correlating the increase in particle count with the increasing total concentration of small molecule in solution, we determined a linear relationship between how many small molecules we added and how many aggregates formed. For nicardipine and miconazole, we calculated that each aggregate was composed of about 10^8 monomer small molecules. After calculating the volume of each monomer, we determined the volume we would predict for 10^8 monomers and compared that to the volume we measured by flow cytometry. Surprisingly, these two volumes were remarkably close, within two-fold error. This suggested that aggregates are densely-packed, solid particles.

One theme that carries across Chapter 1 and 2 is the remarkably tight interaction between aggregate and enzyme as shown by the low off rate observed in Chapter 1 and the stoichiometric inhibition of nanomolar enzyme concentrations by femtomolar concentrations of aggregate particles in Chapter 2. This work provides additional insight into the potential behavior of aggregation in a biological context, as it suggests that aggregates will dissociate upon dilution, returning to their small molecule components once put into a large enough volume. This work was published in the *Journal of the American Chemical Society* in the summer of 2008.

Chapter 2.

2. Stoichiometry and physical chemistry of promiscuous aggregate-based inhibitors

*Kristin E.D. Coan and Brian K. Shoichet**

Department of Pharmaceutical Chemistry & Graduate Group in Chemistry and Chemical Biology

1700 4th St., University of California at San Francisco, San Francisco, California 94158-2550

Abstract

Many false positives in early drug discovery owe to non-specific inhibition by colloid-like aggregates of organic molecules. Despite their prevalence, little is known about aggregate concentration, structure, or dynamic equilibrium; the binding mechanism, stoichiometry with, and affinity for enzymes remain uncertain. To investigate the elementary question of concentration, we counted aggregate particles using flow cytometry. For seven aggregate-forming molecules, aggregates were not observed until the concentration of monomer crossed a threshold, indicating a "critical aggregation concentration" (CAC). Above the CAC, aggregate count increased linearly with added organic material, while the particles dispersed when diluted below the CAC. The concentration of monomeric organic molecule is constant above the CAC, as is the size of the aggregate particles. For two compounds that form large aggregates, nicardipine and miconazole, we measured particle numbers directly by flow cytometry, determining that the aggregate concentration just above the CAC ranged from 5 to 30 fM. By correlating inhibition of an enzyme with aggregate count for these two drugs, we determined that the stoichiometry of binding is about 10,000 enzyme molecules per aggregate particle. Using measured volumes for nicardipine and miconazole aggregate particles (2.1×10^{11} and 4.7×10^{10} $Å^3$, respectively), computed monomer volumes, and the observation that past the CAC all additional monomer forms aggregate particles, we find that aggregates are densely packed particles. Finally, given their size and enzyme stoichiometry, all sequestered enzyme can be comfortably accommodated on the surface of the aggregate.

Introduction

High-throughput screening hit lists are dominated by false positives, many of which are due to promiscuous inhibition by colloid-like aggregates of the small molecule.[11-13, 24, 25, 40, 41] These sub-micron particles form in aqueous media, leading to sequestration and non-specific inhibition of enzymes.[16] In a recent high-throughput screen, 95% of the hits showed signature aggregate behavior, representing the predominant mechanism of false positives and dwarfing the hit rate for non-aggregating, potentially specific inhibitors.[17] Such aggregation is not limited to molecules in screening libraries, as biological reagents and even drugs can also aggregate *in vitro*.[19, 20, 28-33] The potential relevance of aggregation to drug delivery has been suggested by Frenkel et al., who proposed that aggregation facilitates the uptake of non-nucleoside reverse transcriptase inhibitors through absorption by particle-recognizing M cells in Peyer's patches of mucosa-associated lymphoid tissue.[21] Also, aggregates appear to be stable in more biological milieus, such as at high protein concentrations and in cell culture.[18, 42] In short, inhibition by aggregation is a common property among biologically interesting organic molecules with widespread effects in biological systems of varying complexity.

Despite their prevalence, the basic principles of aggregate formation and activity remain obscure. The signatures of aggregate behavior are well known: reversal by non-ionic detergent, steep dose-response curves, and acute sensitivity to enzyme concentration, which have assisted the rapid identification of aggregate-based inhibition.[23, 34, 38, 39, 43, 44] In contrast, we have had no way to measure something as elementary as the concentration of the aggregate particles. Were we able to measure this concentration, we could begin to explore several fundamental properties of aggregates.

What is the stoichiometry of the aggregate-enzyme interaction? Are aggregates in equilibrium with soluble monomer? Are aggregates hollow or solid? Addressing these questions is essential to understanding aggregate structure and mechanism.

Here we measure particle concentrations for known aggregating molecules, using flow cytometry to count the number of particles in a specific volume. By correlating enzyme inhibition with aggregate count, we estimate the stoichiometry and affinity of the binding interaction. We further investigate the point at which aggregates begin to form, the "critical aggregation concentration" (CAC), showing that aggregate count increases linearly with added monomer above the CAC. To determine whether monomer concentration is constant above the CAC, analogous to a critical micelle concentration, we quantified the amount of compound in the aggregate and monomer forms, which were separated by centrifugation. In combination with size measurements and computed volumes for the individual monomers, we deduce that aggregates are densely packed particles. These results expand our fundamental understanding of aggregate behavior and provide insight into the structure, affinity, and mechanism of this ubiquitous inhibitory species.

Experimental

Materials. AmpC β-lactamase was expressed and purified as previously described.[37] Tetraiodophenolphthalein (TIPT), nicardipine, staurosporine aglycone (K252c), latex beads at 110 nm, 200 nm, 310 nm, 460 nm, and 620 nm and Triton X-100 were purchased from Sigma-Aldrich. Miconazole was purchased from ICN and L-755,507 from Tocris. Trifluralin and cinnarizine were a gift of Dr. Joe Goodwin at BD

Biosciences. AlignFlow flow cytometry beads at 2.5 μm were purchased from Invitrogen. Centa was purchased from CalBiochem. All materials were used as supplied by the manufacturer.

Flow Cytometry. Particle characterization was performed using a BD GentestTM Solubility Scanner, a flow cytometer optimized for the detection of insoluble particles. Mixtures were made in a 96-well plate with a final volume of 200 μL per well. All buffers were filtered with a 0.22 micron filter prior to use. Aggregating compounds were diluted into 50 mM potassium phosphate (KPi), pH 7.0, at room temperature, from concentrated stocks in DMSO. For TIPT, K252c, cinnarizine, and trifluralin the final concentration of DMSO was 0.5%. For L-755,507, the final concentration of DMSO was 1%. To reduce error, solutions of 50 μM nicardipine and 10 μM miconazole in 0.1% DMSO were made and serially diluted into 50 mM KPi buffer containing 0.1% DMSO. All solutions were mixed gently by pipette approximately five seconds after the addition of compound and measurements were begun immediately thereafter. Each concentration was measured in at least triplicate. The background count of buffer with DMSO in the absence of compound was measured for each data set and this value was subtracted from each measurement in the set. The counting accuracy of the flow cytometer was calibrated using latex beads at the following sizes: 110 nm, 200 nm, 310 nm, 460 nm, 620 nm, and Nile Red calibration beads at 2.49 μm. We compared the known bead concentration to that measured by the flow cytometer and determined that for particles with size distributions above 300 nm in diameter, the flow cytometer undercounted the bead concentration by 2.85-fold (the average error for beads greater than 300 nm). To account for this undercounting, all particle counts were multiplied by a correction factor of 2.85.

For particles below 300 nm, the counting accuracy of the flow cytometer deteriorated rapidly and so we estimated an upper limit based on a correction factor of 2,000. This factor was determined based on the maximum error observed (1,000-fold at 100 nm) and the fact that we only observed about half of the size distribution, since the flow cytometer does not measure particles below 100 nm. Measurements were acquired for three seconds with a flow rate of 0.5 μL per second using a 3 mW laser at 635 nm. Photon signatures were collected at 90° with a PMT setting of 99 and the threshold channel was set to 25.

β-lactamase Assays. β-lactamase activity and inhibition were monitored in 50 mM KPi buffer, pH 7.0, at room temperature. The substrate centa was prepared as a 12.5 mM stock in 50 mM KPi buffer. DMSO stocks of aggregating inhibitors nicardipine and miconazole were prepared so that the final concentration of DMSO was 0.1%. IC_{50} values for the other compounds were measured at the indicated concentrations of DMSO (Table 3). Each concentration of inhibitor was measured in triplicate for miconazole and nicardipine, and duplicate for the rest of the compounds. Results were controlled for the effect of DMSO on enzyme rates. Inhibitor and 2.3 nM β-lactamase were incubated for five minutes and the reaction was initiated by the addition of 125 μM centa substrate. Change in absorbance was monitored at 405 nm for 100 seconds.

Dynamic Light Scattering. The aggregators miconazole and nicardipine were delivered from concentrated DMSO stocks into filtered 50 mM KPi buffer, pH 7.0 at room temperature for a final concentration of 0.1% DMSO. Measurements were made using a DynaPro MS/X with a 55 mW laser at 826.6 nm, using a detector angle of 90°. The laser power was 100% and the integration time was 200 seconds. Data was filtered using a maximum sum of squares difference (SOS) of 100 and a baseline limit of 1 ±

0.01. Histograms represent the average of three independent data sets, each with at least ten measurements.

Determination of Monomer and Aggregate Concentrations. Solutions of nicardipine, K252c, and TIPT were prepared at multiple concentrations by diluting concentrated DMSO stocks into 50 mM KPi, pH 7.0. The final amount of DMSO at each concentration was 0.1% for nicardipine, 0.5% for K252c, and 5% for TIPT. Solutions were mixed gently and then centrifuged at 16,000 x g for one hour. After centrifugation, the supernatant was collected and analyzed by flow cytometry to determine that there were no detectable aggregates remaining in solution. The bottom 10 μL (from a starting volume of 1 mL) were considered the pellet and resuspended in pure DMSO. UV-visible spectrophotometry was used to determine the concentration of compound in the supernatant and the resuspended pellet. Each concentration was prepared and measured in triplicate. Absorbance was measured at 355 nm for the nicardipine supernatant (0.1% DMSO in 50 mM KPi, $\varepsilon = 6.3x103\ M^{-1}cm^{-1}$) and 352 nm for the pellet (1% KPi in pure DMSO, $\varepsilon = 6.0x103\ M^{-1}cm^{-1}$). Absorbance was measured at 372 nm for the K252c supernatant (0.5% DMSO in 50 mM KPi, $\varepsilon = 5.5x103\ M^{-1}cm^{-1}$) and 335 nm for the pellet (1% KPi in pure DMSO, $\varepsilon = 1.8x104\ M^{-1}cm^{-1}$). The TIPT supernatant was lyophilized overnight and resuspended in 1% KPi in pure DMSO. Absorbance was measured at 337 nm for the TIPT supernatant and pellet (1% KPi in pure DMSO, $\varepsilon = 1.3x104\ M^{-1}cm^{-1}$).

Calculations. The Mitools toolkit (Molinspiration.com) was used to calculate the molecular volume for each compound, which was multiplied by the number of monomers per aggregate to determine a calculated volume for the aggregate. The dimensions of a

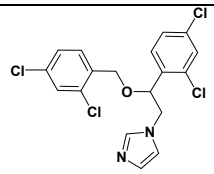
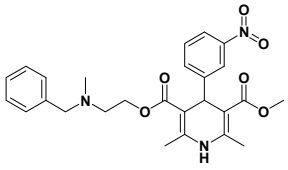
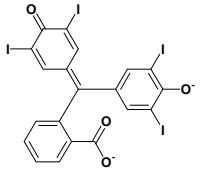
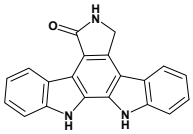
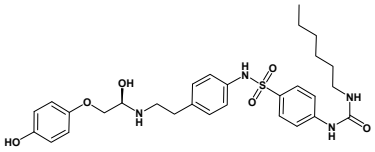
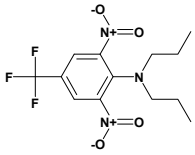
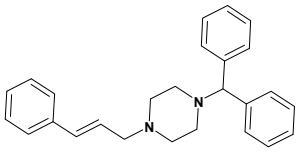
molecule of AmpC β-lactamase were determined using PyMOL and PDB structure 1KE9.

Results

To determine the concentration of the aggregate species, we counted aggregate particles in a known volume using the BD Gentest™ Solubility Scanner, a flow cytometer specifically optimized for counting and characterizing aggregate particles using light scattering.[45] We chose seven known aggregators: miconazole, nicardipine, tetraiodophenolphthalein (TIPT), staurosporine aglycone (K262c), L-755,507, cinnarizine, and trifluralin (Table 3). For each compound, we tested a range of concentrations until we began to observe particle formation, indicated by an increase in the intensity of scattered light. Once the total concentration of small molecule passed this threshold (the CAC), we observed a sharp and linear increase in the number of particles (Figure 7). For instance, below 30 μM nicardipine, an insignificant number of particles were detected by the flow cytometer (Figure 7a). Above 32 μM of the monomer, the number of particles increased steadily at a rate of nearly 10,000 particles for every 5 μM increase in the concentration of the organic molecule (in a volume of 1.5 μL). To minimize error, these concentrations were created by serial dilution; however, similar relationships were observed when each concentration was made individually (data not shown). As a practical note, the CAC could vary dramatically for a given molecule depending on the composition of the buffer, particularly with respect to the DMSO concentration. In extreme cases, we observed as much as a 10-fold change in the CAC.

For example, the CAC for TIPT went from ~1 μM to ~10 μM when going from 0.1% to 1% DMSO (data not shown).

Table 3. Characteristics of aggregating inhibitors measured by flow cytometry.

Compound		IC_{50} vs. β-lactamase (μM)	CAC (μM)	Total Organic Molecule (μM)	Aggregate Concentration (fM)	Diameter (nm)
Miconazole		25[a]	3 ± 2	4-9	5-27	394
Nicardipine		66[a]	32 ± 3	34-44	6-28	569
TIPT		5[b]	10 ± 2	NM[d]	NM[d]	140
K252c		8[b]	4 ± 2	NM[d]	NM[d]	150
L-755,507		3[c]	6 ± 2	6-11	<10 pM	150
Trifluralin		7[b]	3 ± 2	2-5	<10 pM	140
Cinnarizine		40[b]	7 ± 3	7-10	<10 pM	150

[a] 0.1% DMSO. [b] 0.5% DMSO. [c] 1% DMSO. [d] Not measured.

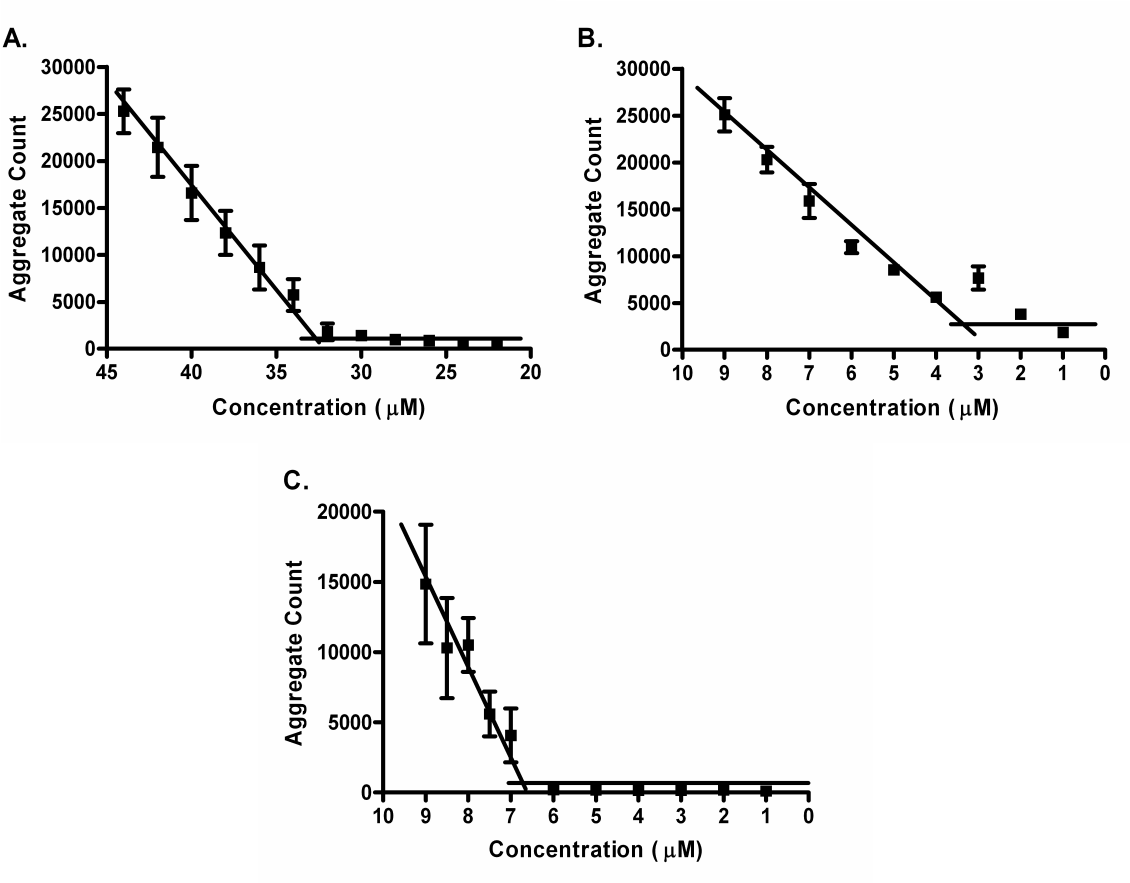


Figure 7. Critical aggregation concentrations. Critical aggregation points of (a) nicardipine at 0.1% DMSO, (b) miconazole at 0.1% DMSO, and (c) L-755,507 at 1% DMSO in 1.5 μL of 50 mM potassium phosphate buffer measured using flow cytometry. For (a) and (b), solutions were made by serial dilution, while compound was added directly for (c). Concentrations are represented as the mean and standard deviation of at least three replicates.

Simply using the known volume of the sample, we used these particle counts to calculate the particle concentration. For nicardipine and miconazole, which form particles between 300 and 600 nm in diameter, we found that the particle concentration just above the CAC ranged from 5 to 30 fM, nearly 10^9-fold lower than the concentration of the monomeric small molecule (Table 3). For smaller aggregates, such as those composed of trifluralin, cinnarizine, TIPT, and L-755,507 (with diameters around 150 nm), the particle count increased much more rapidly, quickly surpassing the detection limits of the instrument and preventing more detailed analysis. In addition, for particles sized below 300 nm in diameter, the flow cytometer grossly undercounts the number of particles. We therefore multiplied the observed number of particles by a factor of 2,000 to estimate the upper limit for the particle count, resulting in low picomolar concentrations. Whereas we believe the concentrations of miconazole and nicardipine aggregates to be accurate, those for the smaller particles must be considered conservative estimates.

Knowing these particle concentrations enabled us to determine the stoichiometry of the aggregate-enzyme interaction for the two aggregators, nicardipine and miconazole, for which we had accurate concentrations. By assuming that percent inhibition directly correlates to the percent of bound enzyme, we related the number of bound enzyme molecules to the number of aggregate particles counted by the flow cytometer (Figure 8). To do so, we measured the inhibition of a model enzyme, AmpC β-lactamase, in the presence of directly measurable concentrations of aggregate particles. We chose nicardipine and miconazole because the concentration of particles increased slowly enough to allow a linear analysis of the particle count versus percent inhibition. Even for nicardipine and miconazole, these aggregate concentrations corresponded only to

inhibition that ranged from zero to about 30%. To estimate the aggregate concentration at the IC_{50}, we extrapolated the ratio between added organic molecule and particle concentration (Figure 8). Since inhibition is linearly related to the concentration of organic molecule and there is a linear relationship between inhibition and particle count through 30% inhibition, we assume that a linear model is adequate for extrapolating the particle count. Over the measured concentrations, the slopes of the inhibition versus particle graphs represent the number of molecules of enzyme bound per aggregate: 12,850 ± 875 for nicardipine and 10,040 ± 440 for miconazole.

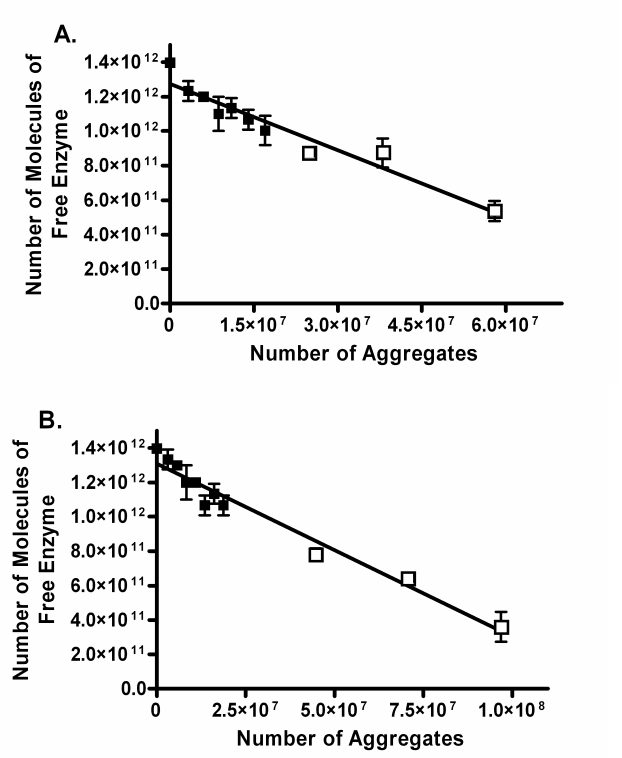


Figure 8. Correlation of β-lactamase inhibition to aggregate count. Correlation of β-lactamase inhibition to aggregate count for (a) nicardipine and (b) miconazole at 0.1% DMSO. The number of molecules of free enzyme is calculated as the product of percent activity and the total amount of enzyme in 1 mL. Aggregate count is measured by flow cytometry in a volume of 1.5 μL and extrapolated for 1 mL. Empty boxes represent aggregate counts that were extrapolated based on a linear regression analysis of the measured aggregate count data points because the count was above the detection limit of the flow cytometer at higher concentrations of aggregating molecule. Free enzyme values are represented as the mean and standard deviation of three replicates. Aggregate counts are the mean of at least five measurements.

One long-standing question has been whether enzyme is adsorbed to the aggregate surface or absorbed into the aggregate. To address this, we next determined whether the calculated number of bound enzyme molecules could even fit upon the surface of the aggregate. If the surface area is insufficient to accommodate all of the bound enzyme, this would suggest that absorption into the aggregate is necessary. To be conservative, we chose the smallest principle dimension of the model protein to calculate the volume of a shell around an aggregate, representing the minimum volume available to surface-bound enzyme (the smallest principle dimension of AmpC is 3.7 nm). We calculated the volume of a 3.7 nm shell around an aggregate of nicardipine or miconazole. We then compared this volume to the total volume of 12,850 or 10,040 enzyme molecules (for nicardipine and miconazole, respectively), modeled as rectangles with dimensions equal to the principle dimensions of an AmpC molecule. When we compared these two volumes, we found that there was more than enough room to accommodate all enzyme molecules on the surface of the aggregate. The total volume of enzyme bound is only 16% of the total volume available in a 3.7 nm shell around a nicardipine particle (diameter 569 nm), and 26% of a shell around a miconazole particle (diameter 394 nm).

In measuring the CAC, we found that aggregation is fully reversible if the aggregates are diluted below their CACs (Figure 7). For example, at 40 μM nicardipine, there are approximately 16,000 particles; however, at half that concentration, 20 μM, the count has returned to the background count around 1,000, showing that the aggregates will dissociate if diluted below the CAC (~33 μM for nicardipine). This reversibility resembles micelle behavior, and so we looked for other behavior we would expect based

on micelle theory.[46, 47] For micelles, the linear increase in particle count beyond the CMC is due to two things: (a) the concentration of free monomer will never go higher than the CMC and all monomeric molecules added beyond the CMC will form micelles and (b) the micelles formed are uniform in their size distribution across increasing concentrations.

To investigate monomer concentration above the CAC, we looked at three compounds that had relatively strong UV-visible absorbance spectra: nicardipine, K252c, and TIPT. To quantify the monomer and aggregate phases, we centrifuged aqueous solutions of varied concentrations of aggregating molecules and separated the supernatant and pellet. After one hour of centrifugation, no aggregates were detectable in the supernatant, as measured by flow cytometry, indicating that the supernatant represented the monomeric fraction. The pellet (representing the aggregate fraction) was resuspended in DMSO and the concentrations of monomer (supernatant) and aggregate (pellet) were measured spectrophotometrically. For each compound tested, the concentration in the supernatant remained constant above the CAC while the aggregate concentration (the pellet) increased steadily, suggesting that all additional compound above the CAC forms aggregates (Figure 9). We also found that the size distribution of the particles remained fairly constant as the concentration of compound was raised above the CAC, at least to the detection limit of the instrument (Figure 10). Dynamic light scattering confirmed that there was no significant population under 100 nm, which is below the detection limit of the flow cytometer (Figure 10 insets).

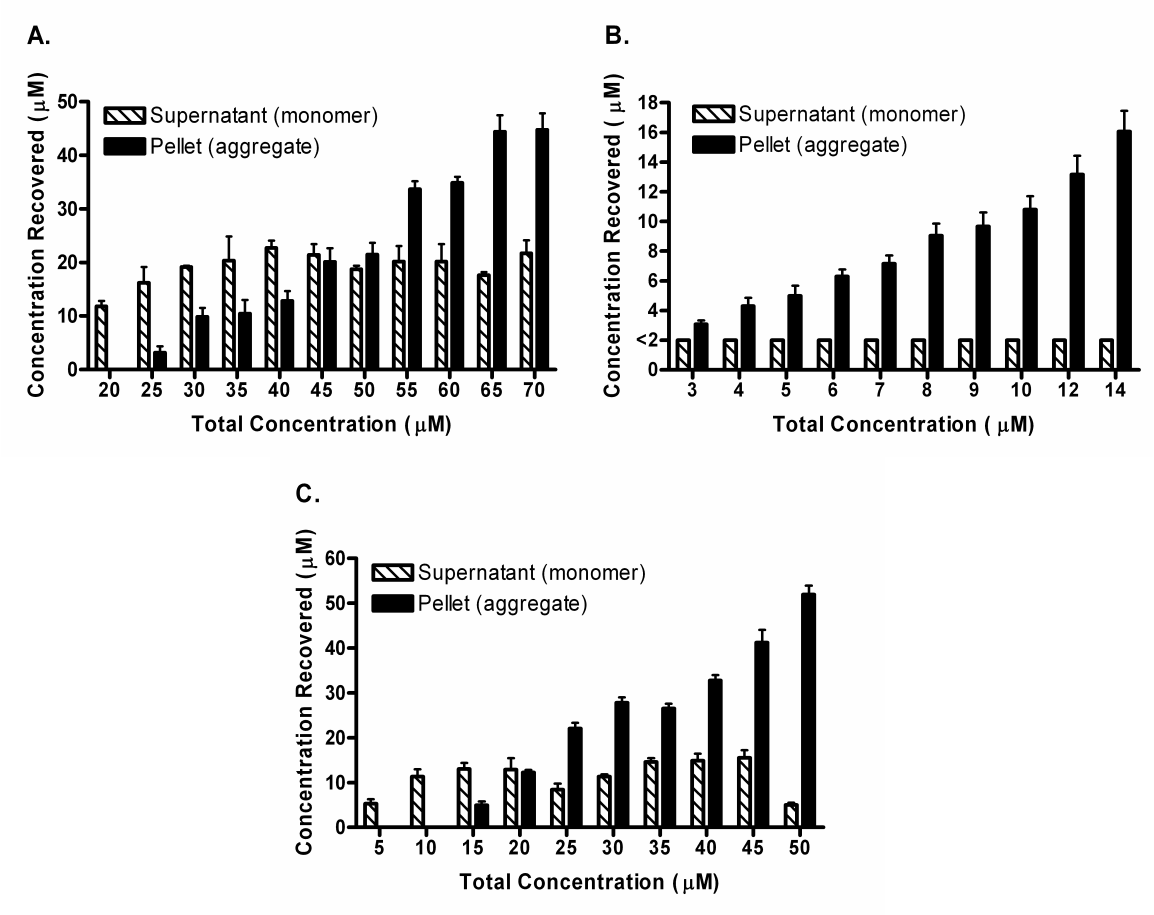


Figure 9. Concentration of monomer and aggregate fractions. Concentration of monomer and aggregate fractions in (a) nicardipine at 0.1% DMSO, (b) K252c at 0.5% DMSO, and (c) TIPT at 5% DMSO in 50 mM KPi buffer. Aggregates were pulled down by centrifugation at 16,000 x g for 1 hour. The supernatant was removed and the concentration of soluble monomer was determined by UV-visible spectrophotometry. The concentration of compound in the aggregate form was determined by resuspending the pellet in DMSO and spectrophotometric analysis. Bars represent the mean and standard deviation of three replicate measurements.

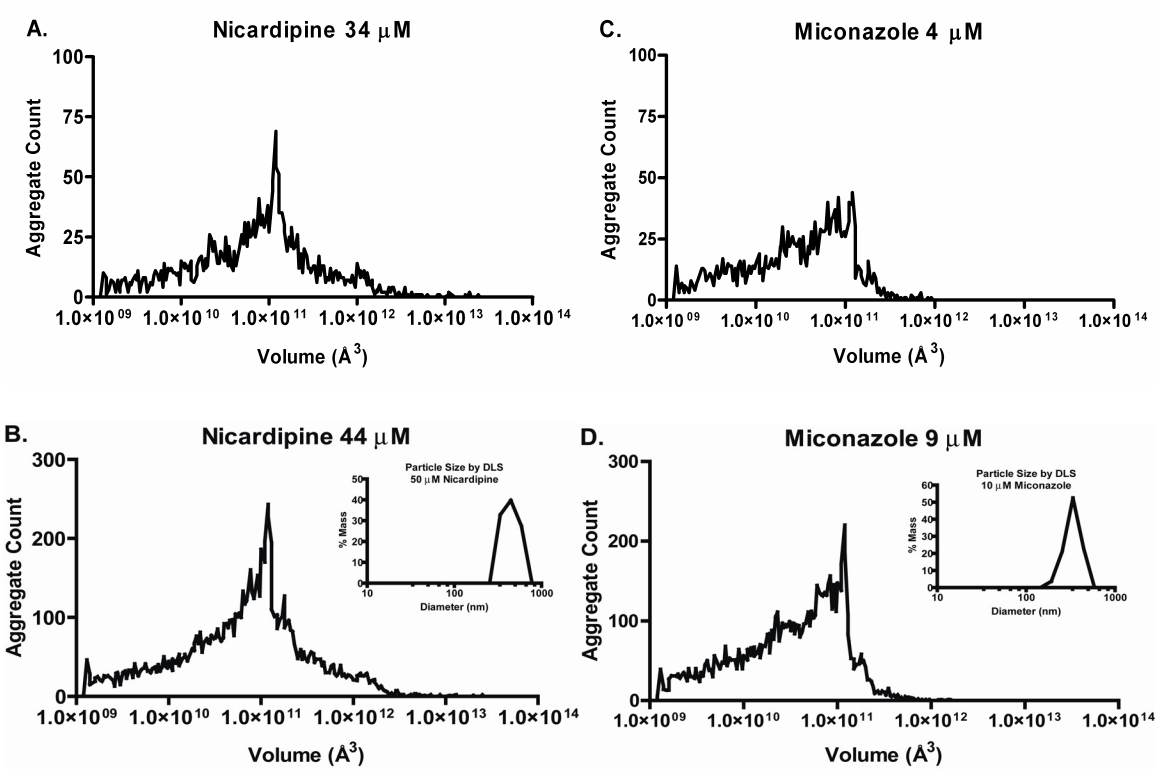


Figure 10. Size distribution histograms. Size distribution histograms of (a) 34 μM nicardipine, (b) 44 μM nicardipine, (c) 4 μM miconazole, and (d) 9 μM miconazole determined by flow cytometry. Dynamic light scattering confirmed that there were no particles below 100 nm in diameter, which is below the detection limit of the flow cytometer (inset of b and d). All samples are in filtered 50 mM KPi, 0.1% DMSO.

Since aggregate count increased linearly and all monomer beyond the CAC forms aggregates, we could calculate the number of monomers that form each aggregate. For accuracy we again focused on miconazole and nicardipine, which form the largest particles. We graphed the total concentration of small molecule monomer added versus particle count over the linear range (Figure 7a and b). The resulting slopes represent the average number of monomers that form each aggregate particle: $4.5x10^8$ monomers per particle for nicardipine and $2.3x10^8$ monomers per particle for miconazole. Since we knew the sizes of these aggregates by light scattering, and we could calculate the volume of each monomer of organic molecule, we could then calculate how the volume of this number of monomers compared to the measured volume. We divided the particle volumes measured by flow cytometry ($V_{measured}$) by the calculated total volume of monomers in each aggregate ($V_{calculated}$, calculated monomer volume multiplied by number of monomers per aggregate) (Figure 11). Here, $V_{calculated}$ is an estimate of the size of a perfectly packed aggregate, a minimum volume for a solid aggregate. For nicardipine the ratio of $V_{measured}$ /$V_{calculated}$ was 1.1, and for miconazole the ratio was 0.6. These ratios indicate that the integrated volume of the monomers that make up each particle is sufficient to account for most or all of the measured volume of the particle, suggesting that these aggregates are solidly packed with monomer and inconsistent with the possibility that they might be hollow.

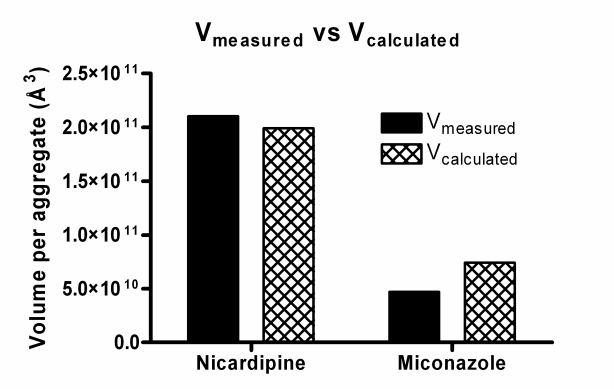


Figure 11. Calculated and measured aggregate volumes. Comparison of calculated aggregate volumes to measured volumes for nicardipine and miconazole. Calculated volumes are the product of the molecular volume from Mitools and the predicted number of monomers that form each aggregate. The measured volume is the mean of the size distribution obtained using flow cytometry.

Discussion

Non-specific inhibition by aggregation is widespread in early drug discovery, but the properties and mechanism of these aggregates are poorly understood. An impediment has been our inability to simply measure aggregate concentration; overcoming this problem is a key result of this work. The particle concentration just beyond the critical aggregation point is in the low femtomolar range (Figure 7, Table 3). From this observation several others follow. First, we investigate the stoichiometry of the enzyme-aggregate interaction and the implications of this to the binding affinity and mechanism. Second, by correlating the amount of added organic material to the rise in particle number, we find that large aggregates are composed of 10^8 organic monomers, the total volume of which closely matches the measured particle volumes, suggesting the particles are solid (Figure 11). Finally, we elucidate some of the basic characteristics of aggregate formation, most notably that aggregation is reversible, and in this respect it resembles micelle behavior (Figure 9 and 10).

By correlating enzyme inhibition to particle concentration, we found that larger aggregates, such as miconazole and nicardipine, bound over ten thousand enzyme molecules per aggregate (Figure 8). As outlandish as this stoichiometry is, it is consistent with what we know about aggregate behavior. A distinguishing feature of aggregate-based inhibition is its sensitivity to changes in enzyme concentration. From early studies we knew that increasing enzyme concentration ten-fold eliminated the observed inhibition, which seemed unprecedented for an inhibitor present at concentrations 1,000 to 10,000-fold higher than the enzyme.[16, 34] Since the stoichiometry of enzyme to aggregate is now shown to be roughly 10,000 to 1, and since the aggregates themselves

are present only in the mid-femtomolar range, increasing enzyme concentration simply overwhelms the capacity of the aggregate to sequester enzyme, eliminating inhibition. By first principles, the observation that inhibition is stoichiometric in the low nanomolar concentration range for both enzyme and enzyme-binding sites on the aggregate suggests that the K_d of this interaction must be at least two orders of magnitude, and more likely three, below these concentrations. Both the high molar ratio and high affinity are consistent with the steep dose-response curves that are often associated with aggregate-based inhibition[17, 39] and the low off-rate of enzyme from the aggregates.[42]

Despite this remarkable stoichiometry, we calculate that even ten thousand enzyme molecules can fit comfortably on the surface of the aggregate, supporting an adsorption model for binding. Using transmission electron microscopy (TEM), we have previously observed enzyme adsorbed to the surface of aggregate particles; however, it is difficult to determine whether enzyme molecules are absorbed inside the aggregate. Although we still cannot rule out absorption, an adsorption mechanism is consistent with our observations that low concentrations of non-ionic detergent can reverse inhibition without disrupting the aggregates themselves and our finding here that aggregate particles are densely packed.

We found that larger aggregates, like those composed of nicardipine or miconazole molecules, contained approximately 10^8 small molecule monomers per aggregate. To our surprise, the calculated total volume based on the number of small molecules per aggregate closely resembled the observed volumes, suggesting the particles are solid (Figure 11). The calculated volume based on the number of molecules per monomer is an estimated minimum, an unrealistic value that assumes perfect packing,

yet our measured volumes are within two-fold of this value. If these particles were hollow, similar to a liposome, we would expect roughly a 10-fold difference between the measured and calculated volumes. Even with errors from compound sticking to the plate and the small percentage of the particles that fell below the accurate counting range of the flow cytometer, both of which reduce the apparent density of the particles, these results suggest that the aggregates are largely solid.

Several physical properties of these promiscuous aggregates also come into focus. It is easy to imagine that these particles are an intermediate form of precipitate, but that does not seem to be the case. Although aggregates can transition to precipitant as concentration is raised, the latter does not sequester protein.[23, 39] Consistent with these observations, the particles here appear to be in equilibrium with monomer and are a reversible phase. On lowering the concentration of a suspension of aggregates below its CAC the particles rapidly re-dissolve (tens of seconds). As anyone who has tried to dissolve organic material into aqueous solution can attest, this is rarely true for precipitated material, which is why most organic molecules are delivered to aqueous buffer from DMSO stocks. Thus, although the aggregates are only transiently stable, the individual particles appear to approach true equilibrium.

It is appropriate to mention several caveats and remaining gaps. Particle counts made by the BD flow cytometer are only quantitatively reliable for particles in the 400 nm diameter range and larger, and even here require adjustment against controls. The particle count for smaller particles can only be grossly estimated. Similarly, the stoichiometry of the enzyme-aggregate interaction could only be measured in the early part of the inhibition curve, beyond which we exceed the counting capacity of the

instrument. Thus, these results rely on extrapolation, assuming a constant linear correlation. Also, it is possible that not all aggregate-based inhibitors have the same properties and structures as those we have studied here. It is convenient to assume that all promiscuous aggregates will have similar structures and mechanisms, but there is no strong theory to suggest that this is true. Finally, key questions remain unaddressed for aggregate mechanism, including why enzyme becomes inhibited when bound to an aggregate.

These caveats, while important, do not diminish our confidence in the main conclusions of this study, which suggest the following model (Figure 12). At micromolar concentrations, organic molecules can reversibly associate into colloid-like particles in aqueous media. For larger particles, about 10^8 small molecule monomers associate per particle. These particles are densely packed and, again for larger particles, sequester about 10^4 enzyme molecules each. Whereas we cannot rule out the possibility that enzyme is absorbed inside the aggregate, adsorption to the surface is sufficient to accommodate all bound enzyme. This model, though still crude, provides a foundation for future studies into these ubiquitous, but often confounding particles.

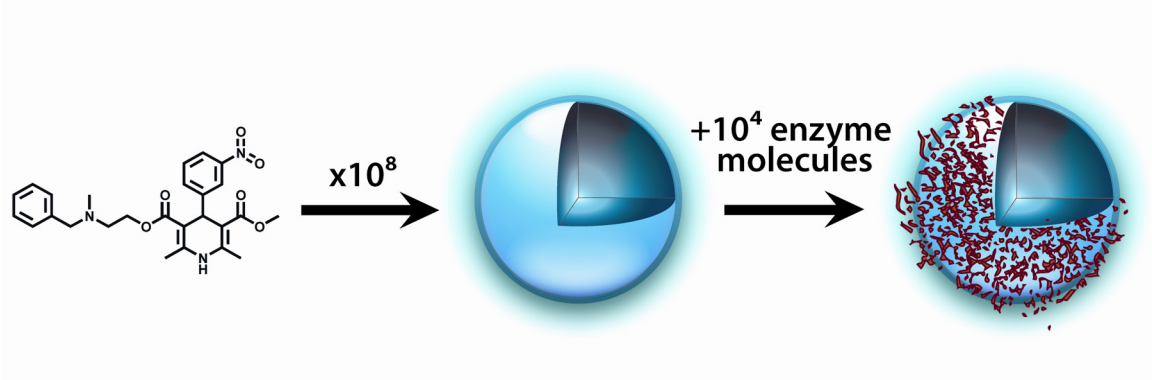


Figure 12. A model of aggregate structure and enzyme binding. Some organic molecules can form densely packed particles (10^8 small molecules per aggregate for larger particles) in aqueous media. Once formed, these larger particles sequester and then inhibit enzyme with a stoichiometry of approximately 10^4 enzyme molecules per aggregate. The surface of the aggregate is sufficient to accommodate all bound enzyme.

Acknowledgements

This work was supported by NIH grant GM71630 (to BKS). KEC was partly supported by NIH grant T32-GM64337 (C. Craik, PI) and by a fellowship in the field of Pharmaceutics awarded by the PhRMA Foundation. We thank Dr. J. Goodwin at BD Biosciences for the generous loan of the BD Gentest™ Solubility Scanner and for helpful discussions. We also thank A. McReynolds for β-lactamase purification, D. Teotico for PyMOL calculations, J. Irwin and J. Hert for Molinspiration calculations. We also thank R. Ferreira, V. Thomas, D. Teotico, M. Keiser, and J. Irwin for reading this manuscript.

Gloss to Chapter 3.

Another fundamental question about aggregation was to determine why aggregates inhibit enzyme. McGovern *et al.* wrote a paper on this subject, "A specific mechanism of nonspecific inhibition," which advanced our understanding of aggregation considerably.[34] This work elucidated characteristic aggregate behavior, showing it is possible to cosediment enzyme-aggregate complexes by centrifugation, rapidly reverse inhibition by the nonionic detergent Triton X-100, and visualize aggregates bound to enzyme by electron microscopy. In addition, they performed experiments that suggested that aggregates were not causing enzyme denaturation, but the mechanism was unclear. What other effects could the aggregate have that would lead to enzyme inhibition?

It is not readily evident why aggregate-bound enzyme should be inactive. There are examples of enzymes being immobilized to a support where the enzyme retains its activity, such as immobilized pepsin. We proposed three models that might explain inhibition. First, perhaps the enzyme was not fully denatured, but only partly so. Such small scale denaturation would prevent the enzyme from being active, but the enzyme would be able to rapidly refold when released from the aggregate by detergent. Second, binding to the aggregate might rigidify the enzyme, preventing the normal dynamic and catalytic motions. Finally, the aggregate may simply sequester the enzyme and physically block access to the active site.

To dissect which of these mechanisms might be true, we needed a technique that could assess solvent accessibility. We considered covalent probes, proteolysis, and circular dichroism to name a few, but we believed that many of these techniques would

be convoluted by the aggregates themselves. Given the highly conjugated ring systems of most aggregators, we feared the aggregates would fluoresce so much that the protein signal would be lost. In the case of proteolysis, we suspected the aggregates would inhibit the protease as well. Additionally, we did not want residue specific information. Given the nonspecific nature of aggregates, it would be impossible for us to predict which amino acids are affected by aggregate binding, and we would not even expect there to be specific interactions. Rather, we hoped to investigate a general mechanism, which might account for inhibition across many enzymes.

One technique which did not require fluorescence and which would provide information across the enzyme regardless of amino acid identity was hydrogen deuterium exchange. This technique is commonly used to study enzyme folding and protein binding events, where the solvent accessibility of the enzyme changes depending on its state. By incubating an enzyme in a deuterated solvent, exchangeable hydrogens are replaced by deuterium and the amount of deuterium incorporation is a measure of how accessible those hydrogens were to solvent. This type of exchange can be measured either by nuclear magnetic resonance (NMR) or by mass spectrometry. Again, the aggregates might have interfered with NMR by reducing the tumbling time of the enzyme and broadening the enzyme signal beyond possible detection. Although this complication was not insurmountable, at the outset it appeared that mass spectrometry (MS) would be more tractable and that it would have the practical benefit of requiring less enzyme.

We had to modify the standard hydrogen deuterium exchange MS protocol to accommodate the presence of aggregates, including the addition of detergent to disrupt the aggregates and release the enzyme. It was necessary to release enzyme from the

aggregate prior to MS analysis, but after exchange with the deuterated solvent. An ongoing difficulty with these experiments was the recovery of aggregate-bound enzyme from the aggregate at a high enough concentration to be detected by MS. Often the samples produced very weak signals, indicating that there was not enough peptide to be analyzed. The solution to freeing more aggregate-bound enzyme is to add detergent, but detergent also has a strong signal in the mass spectrum that can overwhelm any signal we see from enzyme.

After optimizing the protocol, we obtained a reproducible data set; however, the data were noisy and in some aspects difficult to interpret. Despite the noise, there was a common trait: nearly every peptide in the presence of aggregates showed deuterium incorporation greater than or equal to the peptides without aggregates. These results suggested that aggregates were increasing deuterium exchange, and therefore solvent accessibility, which would be consistent with an unfolding mechanism.

Given the low quality of the MS data, we searched for another method to determine whether aggregates were unfolding enzyme. This led us to reconsider proteolysis. Susceptibility to digestion is a sensitive indication of whether an enzyme is even partly unfolded. We had previously disregarded proteolysis because of the possibility that the protease itself would also be inhibited by the aggregates. After considering our conclusions about saturating aggregates with excess enzyme in Chapter 1, we hypothesized we could use excess protease to saturate the aggregate and ensure that there was free, active protease.

The first aggregate, rottlerin, showed a dramatic effect: rottlerin-bound β-lactamase was significantly digested within 30 minutes. On the other hand, uninhibited β-

lactamase was stable to digestion for many hours. Five out of ten aggregators tested showed similar sensitivity. This may suggest that there is more than one mechanism of aggregate action, but it is also possible that the others did not show this effect because the proportions of aggregate, β-lactamase, and trypsin were not correct or that the aggregates were too insoluble. Additionally, it appeared that trypsin itself was more susceptible to self-degradation in the presence of aggregates, suggesting that this mechanism might be transferable across different enzymes.

Although we were skeptical of the MS results, the increased susceptibility to proteolysis strongly supported that the enzyme was unfolded on the aggregate. Also, both the MS and the protease results are inconsistent with the other two mechanisms. Neither reduced dynamics nor physical sequestration should increase deuterium exchange or proteolysis. In fact, the opposite should be true. We would expect these two mechanisms to reduce exchange and to protect the enzyme from proteolysis. This work has been submitted to the *Journal of Medicinal Chemistry*.

Chapter 3.

3. Promiscuous aggregate-based inhibitors promote enzyme unfolding

Kristin E.D. Coan[a]‡, *David A. Maltby*[b]‡, *Alma L. Burlingame*[b]*, *and Brian K. Shoichet*[a]*

[a]Department of Pharmaceutical Chemistry & Graduate Group in Chemistry and Chemical Biology, University of California – San Francisco, Byers Hall, 1700 4th St. San Francisco, CA 94158-2550

[b]Mass Spectrometry Facility, Department of Pharmaceutical Chemistry, University of California, San Francisco, 600 16th Street, MC 2240, Genentech Hall, Suite N472A, San Francisco, CA 94158-2517

‡Contributed equally.

*Corresponding authors: shoichet@cgl.ucsf.edu, alb@cgl.ucsf.edu

Abstract

One of the leading sources of false positives in early drug discovery is the formation of organic small molecule aggregates, which inhibit enzymes nonspecifically at micromolar concentrations in aqueous solution. A detailed mechanism of this widespread problem is not known. To investigate the mechanism of inhibition with greater precision, we determined changes in solvent accessibility that occur when an enzyme binds to an aggregate using hydrogen deuterium exchange mass spectrometry of peptic fragments of a model enzyme. For one aggregator, rottlerin, and one enzyme, AmpC β-lactamase, our results indicated that binding to the aggregate led to increased deuterium exchange, suggesting increased solvent accessibility and unfolding as a potential mechanism of inhibition. We reproducibly measured ten peptides covering 41% of the total sequence of β-lactamase, all of which showed deuterium incorporation greater than or equal to that of the enzyme in the absence of inhibitor. To explore whether inhibition was caused by enzyme unfolding, we investigated whether enzyme-aggregate complexes were more susceptible to proteolytic degradation than free uninhibited enzyme. For five aggregators, we saw a significant increase in β-lactamase degradation by the protease trypsin when β-lactamase was digested in the presence of aggregates. Uninhibited enzyme was generally stable to digestion for over four hours, while inhibited enzyme showed significant to complete degradation within fifteen minutes. Combined, these results suggest that the mechanism of action of aggregate-based inhibitors proceeds via enzyme unfolding.

Introduction

Many organic small molecules form submicron aggregates at micromolar concentrations in aqueous solution.[16, 23] Such molecules are found amongst screening hit lists, biological reagents, and even marketed drugs.[18-21, 28-30, 32, 33] These aggregates have the unusual property of nonspecifically inhibiting enzyme targets, leading to false positive "hits" in biochemical assays ─ a problem that is now well-recognized particularly in high-throughput screening.[11-13, 24, 25, 31, 40, 41, 44] Although this is a broadly acknowledged problem, potentially a major contributor to false positives, exactly how aggregates cause inhibition remains poorly understood.[17] Here we revisit the specific mechanism of nonspecific inhibition by investigating the structural changes that are induced in the enzyme upon binding to the aggregate.

In 2003 McGovern *et al*. observed three mechanistic features of small molecule aggregates that guided our investigation.[34] First, inhibition occurs via the direct binding of enzyme to aggregate, as shown by (1) the ability to sediment protein-aggregate complexes with centrifugation, (2) the punctate fluorescence observed by fluorescence microscopy when aggregates are placed in solution with Green Fluorescent Protein (GFP), and (3) the direct observation of protein-aggregate complexes by transmission electron microscopy. Second, a key characteristic of aggregate-based inhibition is that it can be rapidly reversed by the addition of a non-ionic detergent such as Triton X-100, indicating that enzyme can rapidly (within seconds) revert to its active state from its inhibited state on the aggregate. Last, there was an ensemble of experiments which appeared inconsistent with denaturation as a potential mechanism of action. For example, it seemed unlikely that enzyme could rapidly refold into its active state upon the addition

of detergent if it were completely denatured when bound to the aggregate. It seemed equally unlikely that GFP could retain its fluorescence if it were completely denatured while bound to an aggregate. Two other experiments suggested that inhibition was not due to denaturation: (1) additional denaturants such as guanidinium or urea did not increase inhibition by aggregates, if anything inhibition was decreased, and (2) a destabilized enzyme was not found to be any more sensitive to aggregate-based inhibition than its wild type counterpart.

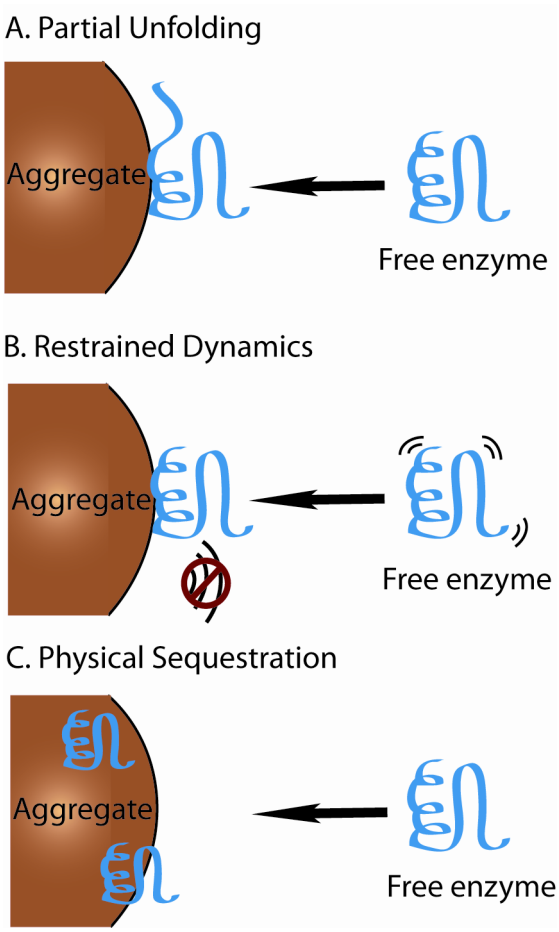


Figure 13. Proposed models for the mechanism of action of promiscuous small-molecule aggregators. (A) Binding to the aggregate promotes a partial unfolding event. (B) Binding to the aggregate constrains protein dynamics and restricts catalytic motions. (C) The aggregate physically blocks the active site and sequesters enzyme away from substrate.

As a result of McGovern's work, we considered three possible mechanisms of action that might explain aggregate-based inhibition (Figure 13). Although we did not believe that there was large scale unfolding of the enzyme, it still seemed reasonable that there might be small-scale or local unfolding events. On the other hand, aggregate binding may have the opposite effect: instead of increasing flexibility, it may rigidify the enzyme, restricting the normal dynamic motions of the enzyme and preventing those movements necessary for catalysis. Finally, aggregates may physically sequester enzyme away from substrate. To explore these potential mechanisms, we chose to use hydrogen deuterium exchange mass spectrometry (HDX), a technique which measures changes in solvent accessibility for processes such as enzyme unfolding or protein-protein interactions.[48-54] HDX mass spectrometry relies on the different exchange rates of the backbone amide protons with a deuterated solvent, which are measured by the change in mass as deuterium replaces hydrogen. To investigate changes in solvent accessibility, we quantified deuterium exchange of AmpC β-lactamase over a time course of eight hours in the presence or absence of an aggregating inhibitor, rottlerin. To obtain localized information, β-lactamase was digested with pepsin after exchange. We reproducibly observed ten fragments covering 41% of the entire enzyme sequence. The differences in solvent accessibility did not seem to be localized to specific regions (given the nonspecific nature of aggregate-based inhibition, we did not expect to see peptide-specific interactions); rather, we observed a general trend across all peptides. The differences in solvent accessibility that we observed by mass spectrometry suggested that we may also see differences in protease sensitivity, which we investigated by gel electrophoresis of tryptic digests of our model enzyme in the presence or absence of

several known aggregating inhibitors. Combined, these experiments suggest small scale enzyme unfolding as a molecular mechanism for aggregate-based inhibition.

Results

To examine the structural changes that occur in an enzyme when bound to a small molecule aggregate, we began by measuring changes in solvent accessibility using hydrogen deuterium exchange mass spectrometry (HDX). We chose AmpC β-lactamase as a model enzyme, which our lab works with routinely, and rottlerin as a model aggregator, because of its relatively high solubility and potency (low micromolar IC_{50} vs. AmpC). β-lactamase was incubated in deuterated MOPS buffer for varied amounts of time between ten minutes and eight hours, either in the presence or absence of rottlerin. After exchange, the aggregates were disrupted with detergent and the exchange reaction was quenched by the addition of cold acid. β-lactamase was subsequently digested with immobilized pepsin to obtain regional exchange information. These peptides were then separated and analyzed by electrospray ionization mass spectrometry coupled with liquid chromatography (ESI LCMS).

Two modifications were necessary to accommodate the presence of aggregates in the HDX experiment. First, aggregate-enzyme complexes had to be exchanged in the deuterated buffer at a lower concentration and then concentrated before further sample preparation. Aggregates are a phase between soluble, free small molecule and precipitant (which does not typically inhibit enzymes) and so there is an upper limit to the concentration that we can take an aggregating inhibitor: the concentration when it ceases

to form more aggregates and instead begins to precipitate. Typically, micromolar concentrations of an aggregating molecule will inhibit nanomolar concentrations of enzyme. These HDX experiments required micromolar concentrations of protein and if a corresponding concentration of inhibitor were used, we would need an inhibitor that was soluble at millimolar concentrations. As aggregation is a form of insolubility, there are very few examples of molecules that have such high solubility and these molecules are often weak aggregators and unsuitable for this analysis.

Since we did not have an aggregator that could be used at millimolar concentrations, we instead reduced the concentration of the enzyme so that it would be mostly inhibited by a soluble concentration of the aggregator (100 μM for rottlerin). We relied on the fact that aggregates and aggregate-bound enzyme can be pelleted, and therefore concentrated, by centrifugation. Although incubation was performed at a lower concentration of enzyme, we could concentrate the enzyme-aggregate complexes prior to analysis by collecting the pellet and removing the supernatant. This process has the additional benefit of guaranteeing that predominantly aggregate-bound complexes would be analyzed, as free enzyme would not be pulled down by centrifugation and would be many-fold lower in concentration.

The second necessary modification was the addition of detergent. Although we tried several detergents, none were nearly as effective as Triton X-100. It was necessary to add Triton mainly to release bound enzyme from the aggregates so that the complexes were not pulled down again when centrifugation was used to remove the immobilized pepsin. This resulted in a delicate balance between using enough detergent to release the enzyme for analysis, but not so much that the detergent signal itself overwhelmed the

peptide signals. To increase separation between the peptide and detergent signals, it was necessary to use ESI LCMS rather than MALDI.[55, 56] As a result of the presence of aggregates, the inhibited samples generally showed much weaker signal intensities compared to uninhibited samples. Often, the signals were so weak that they could not be analyzed. Those that were observed showed considerable noise, probably due to the weak intensities.

The peptides that we reproducibly observed covered 41% of the β-lactamase sequence (Figure 14), representing several regions of the enzyme spanning both buried and exposed regions. Although our results are not strong enough to determine whether specific areas experienced more exchange than others, it was also not our goal to do so. Given that aggregates are nonspecific inhibitors, we were searching for a global effect, a mechanism that could explain inhibition of many enzymes and that was not restricted to specific residues or peptide sequences. The results suggested such a trend. Across all of the peptides that we measured, enzyme-aggregate samples showed deuterium incorporation greater than or equal to the deuterium incorporation of the enzyme alone (Table 4). Levels of deuteration were very low in both samples with and without inhibitor; however, the trend of higher deuterium content in the aggregate-containing samples is consistent across all of the peptides. Two peptides that did show significant exchange are shown in Figure 15 (a complete list of peptides in the Supplementary Material). Again, we observed very low deuterium incorporation, but the time points repeatedly indicated a significant difference between samples with and without aggregates. Those samples containing aggregates consistently show higher deuterium incorporation, suggesting that the enzyme may be unfolded when bound to the aggregate.

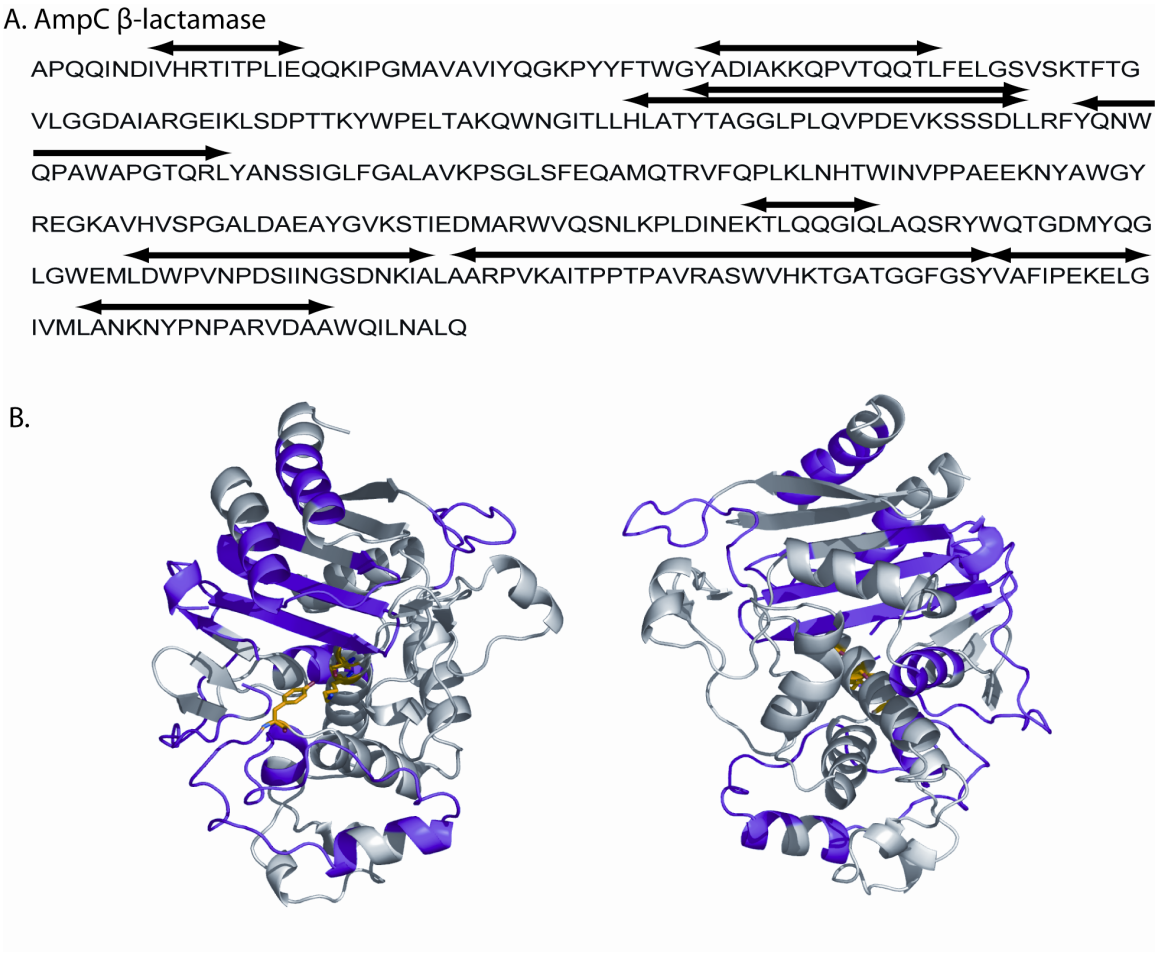


Figure 14. Amino acid sequence and structure of AmpC β-lactamase. (A) Enzyme sequence showing the peptic fragments that could be quantified reproducibly in the mass spectrum both with and without the aggregator rottlerin. (B) Structure of AmpC β-lactamase indicating the location of the peptic fragments that were observed in the mass spectrum (fragments shown in purple, active site residues shown in yellow).[57]

Table 4. Summary of H/D exchange data for AmpC β-lactamase in the presence or absence of the aggregating inhibitor rottlerin.

Peptide	# of amide bonds in peptide[a]	Deuterium Incorporation after 24 hours[b]	Fold increase in deuteration ($D_{inhibited}/D_{uninhibited}$)			Deuterium Incorporation after 8 hours[d]		% solvent accessibility[e]
			10 minute[c]	1 hour[c]	8 hours[c]	no inhibitor	+ rottlerin	
8-18	8	1.8	1.3	2.3	2.5	0.2 ± 0.2	0.5 ± 0.2	35.7
42-56	12	6	1.9	2.7	1.8	0.8 ± 0.2	1.4 ± 0.2	44.7
105-128	20	6.6	1.6	2.6	1.8	1.5 ± 0.6	2.7 ± 0.5	31.3
109-128	16	5.4	1.5	2.5	1.6	1.4 ± 0.4	2.2 ± 0.3	36.9
132-146	11	4.2	2.6	4.2	3.4	0.3 ± 0.1	1.1 ± 0.1	34.8
243-250	6	1.8	1.2	NM[f]	1.7	0.4 ± 0.2	0.7 ± 0.2	20.7
271-289	15	6.6	1.4	2.6	1.4	2.3 ± 0.5	3.1 ± 0.6	40.7
291-322	26	8	1.6	2.5	1.8	1.7 ± 0.6	3.1 ± 0.6	22.3
323-332	7	1	1.3	3.0	2.7	0.2 ± 0.0	0.5 ± 0.1	11.8
336-350	11	2.7	1.7	3.7	2.0	0.5 ± 0.2	1.0 ± 0.2	21.6

[a]Equal to number of amide bonds + 1, excluding prolines.

[b]Deuterium incorporation is calculated by subtracting the mass of the control undeuterated centroid from the centroid of the deuterated sample. Deuterium incorporation is not corrected for back exchange.

[c]Indicates the amount of time the samples were exchanged in the deuterated solvent.

[d]Deuterium incorporation is calculated as the (deuterated mass * charge - charge) minus (undeuterated mass * charge – charge) with the standard deviation of three replicate measurements.

[e]Average ratio of side chain surface area to "random coil" values per residue calculated using Getarea.[58] Greater than 50% is considered exposed and less that 20% is considered buried.

[f]Not measured.

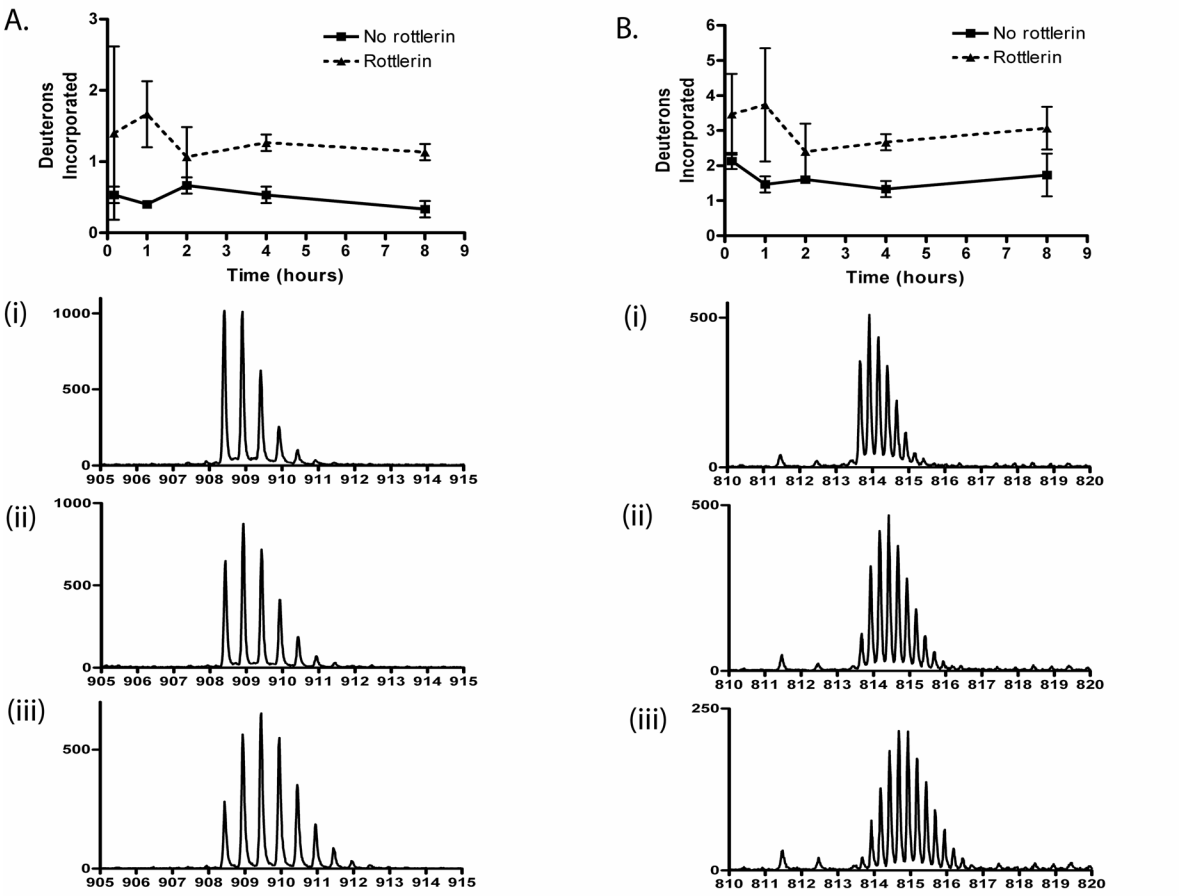


Figure 15. Mass envelopes and corresponding deuterium incorporation plots. Mass envelopes and corresponding deuterium incorporation plots for two fragments from the mass spectrum of β-lactamase in the presence (solid line) or absence (dotted line) of the aggregator rottlerin. The spectra have been expanded to show the isotopic distribution of the ions of interest: (A) the peptide containing residues 132-146 (monoisotopic m/z = 908.4, +2 charge state) and (B) the peptide containing residues 291-322 (monoisotopic m/z = 813.7, +4 charge state). For each peptide, the isotopic distributions are shown for (i) the undeuterated sample, (ii) the sample that was deuterated for four hours in the absence of rottlerin, and (iii) the sample that was deuterated for four hours in the presence of rottlerin.

To investigate whether aggregates were unfolding bound enzyme, we investigated whether aggregate-bound enzyme was more susceptible to proteolytic degradation. If an enzyme is even slightly unfolded, it should be measurably more sensitive to digestion by proteases (Figure 16). Although we had considered this experiment before, we had not tried it due to concerns that the protease itself would be inhibited by the aggregate. Given recent results showing the very slow off rate of enzyme from the aggregate and that the aggregate can be saturated with additional enzyme (in this case excess protease), we reconsidered the feasibility of this experiment.[39, 42, 59] We chose five known aggregators: rottlerin, Congo Red, Eriochrome Blue Black B, nicardipine, and L-755,507. We again used β-lactamase as our model enzyme and chose trypsin as the protease. β-lactamase was pre-incubated with or without aggregates before the addition of trypsin to avoid competition with trypsin for binding space on the aggregate. Free or aggregate-bound enzyme was then incubated with trypsin between zero minutes and four hours and the digestion was monitored by gel electrophoresis.

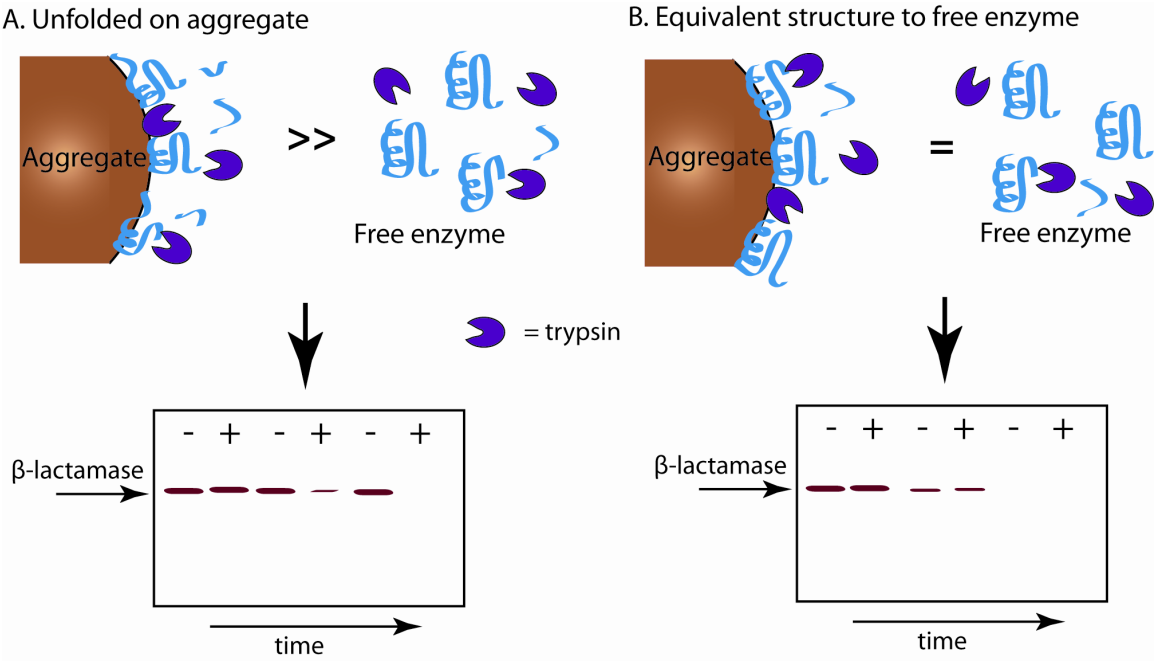
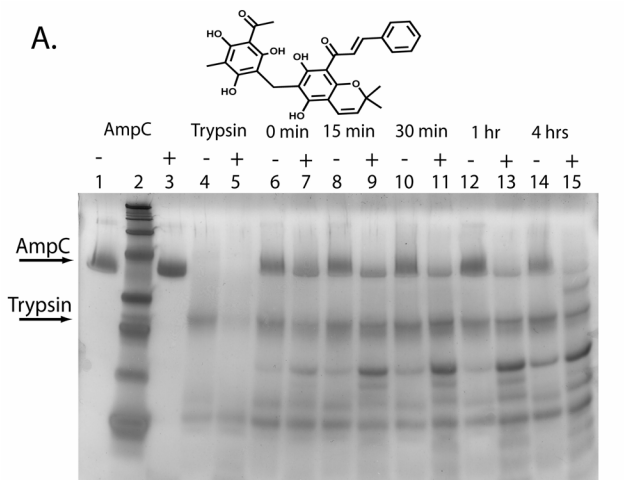
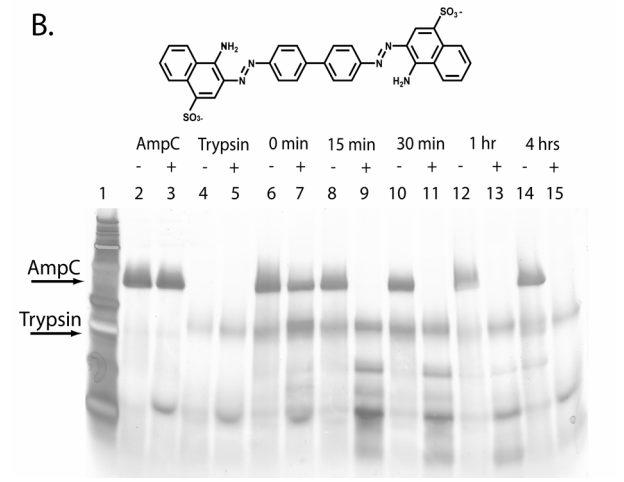
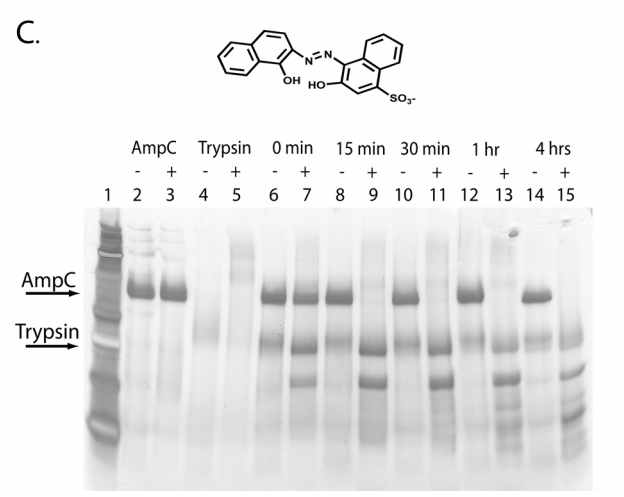


Figure 16. Schematic representation of the tryptic digest experiment and interpretation. If enzyme is unfolded on the aggregate, it should be more susceptible to proteolysis compared to enzyme in the absence of aggregates (A). If the enzyme is in its native state when bound to the aggregate, identical to unbound enzyme, it should be degraded at a comparable rate (B). Although some trypsin may be bound to the aggregate, trypsin will be used in excess so as to ensure that the aggregate is saturated and that there is free, active trypsin in solution. (+) indicates that aggregates are present, (-) indicates free enzyme.

For all of the aggregators, the presence of aggregates had no effect on the band representing β-lactamase in the absence of trypsin (Figure 17, lanes 2 and 3 or 1 and 3). In contrast, when we added trypsin we observed significant digestion in the presence of aggregates, but not in the *absence* (Figure 17). For example, in Figure 17a, the β-lactamase band is present in both samples at zero minutes (lanes 6 & 7), but as time progresses, the β-lactamase band in the presence of rottlerin becomes weaker and weaker until it is completely gone by four hours (lane 15). Conversely, β-lactamase in the absence of rottlerin appears almost as strong after fours hours with trypsin as it did after zero minutes (lane 14). For Congo Red and Eriochrome, the β-lactamase band is present in both samples at zero minutes of trypsin incubation (Figure 17 b and c, lanes 6 and 7), but after only 15 minutes the β-lactamase band is gone in the presence of aggregates (lane 9). Again, β-lactamase in the absence of aggregates remains undigested by trypsin even after 4 hours (lane 14). Although the effect was less pronounced, two other aggregators, nicardipine and L-755,507 also increased sensitivity to trypsin (Fig 17 d and e). In many of these samples, it is also possible to observe the formation of a large degradation product of β-lactamase running below the trypsin band (Figure 17 a-c). Trypsin also appears to experience more self-degradation in the presence of aggregators (Figure 17 a and c, lanes 4 and 5).


A.
AmpC
Trypsin
0 min
15 min
30 min
1 hr
4 hrs
- + - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
AmpC
Trypsin


B.
AmpC
Trypsin
0 min
15 min
30 min
1 hr
4 hrs
- + - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
AmpC
Trypsin


C.
AmpC
Trypsin
0 min
15 min
30 min
1 hr
4 hrs
- + - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
AmpC
Trypsin

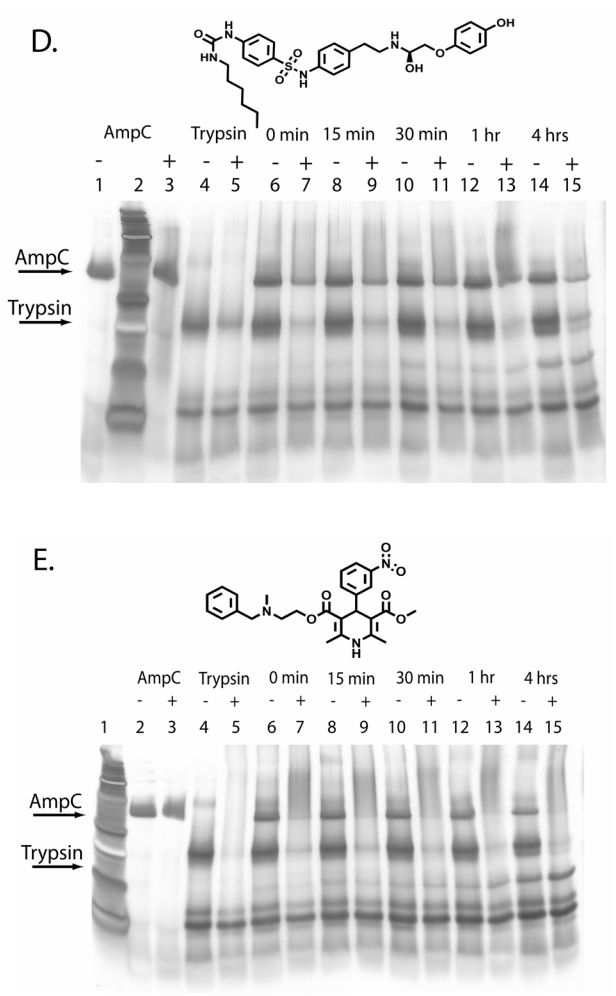


Figure 17. SDS-PAGE and silver stain of tryptic digests of β-lactamase in the presence or absence of aggregating inhibitors. (A) The inhibitor is 100 μM rottlerin, the same inhibitor used in the HDX MS experiments. (B) The inhibitor is 250 μM Congo Red. (C) The inhibitor is 250 μM Eriochrome Blue Black B. (D) The inhibitor is 100 μM L-755,507. (E) The inhibitor is 250 μM nicardipine. For gels B, C, and E, lane 1 is a molecular weight ladder, for gels A and D, lane 2 is a molecular weight ladder. Lanes 2 and 3 for gels B, C, and E or lanes 1 and 3 for gels A and D, show β-lactamase without trypsin, in the absence (-) or presence (+) of aggregator. Lanes 4 and 5 contain trypsin (no β-lactamase) in the absence and presence of inhibitor, respectively. Lanes 6 through 15

contain tryptic digests of β-lactamase in the absence or presence of inhibitor for digestion times of 0 minutes, 15 minutes, 30 minutes, 1 hour, and 4 hours (from left to right). β-lactamase was used at a concentration of 0.5 μM in all experiments. Trypsin was used at a concentration of 0.01 mg/mL for (A), 0.025 mg/mL for (B), (C), and (D), and 0.05 mg/mL for (E).

Discussion

Aggregate-based nonspecific inhibition is undeniably a widespread problem, but further elucidating the mechanism of inhibition has been challenging. In many cases, the typical techniques used for observing changes in protein structure are confounded by the presence of the aggregates themselves: for example circular dichroism, X-ray crystallography, and fluorescence-based techniques like Förster resonance energy transfer. Furthermore, we were not searching for a mechanism that involved specific residues or sequences. Aggregates nonspecifically bind and inhibit enzymes, therefore there should be a global mechanism of action that does not rely on the specific identity of any given enzyme. Parsing out the specific details of a single enzyme-aggregate interaction was not our goal. Rather, we were looking for a general mechanism that could explain the nonspecific action of aggregates.

Although HDX mass spectrometry is often used to determine sequence-specific binding events, the presence of the aggregates proved to be a significant obstacle, which resulted in high levels of noise and troublesome reproducibility. The samples that contained aggregates typically showed very low peptide signal strength, suggesting that we were not effective in disrupting the aggregate-enzyme interaction and we were losing peptide during sample preparation. Our standard solution to this problem is to add more detergent, Triton X-100; however, this posed a serious problem in MS analysis because the detergent peaks could easily overwhelm our peptide signals. We tried several alternatives to Triton, including beta-octa-glucoside, other detergents, and organic solvents like DMSO (which increases the solubility of the free small molecule form of an aggregator). None of these substitutions were successful. It is likely that we also lost a

significant portion of our enzyme during the concentration/centrifugation step, but we were at the solubility limit of rottlerin and other more soluble aggregators proved ineffective at pulling down more enzyme. Taking into account these caveats, it appeared that we could make one observation from our data: we reproducibly saw an increase in solvent accessibility in the presence of aggregates. Furthermore, all peptides that we observed showed equal or greater levels of deuteration in the presence of aggregators. We were skeptical of this result, but it did make us reconsider unfolding as a mechanism on inhibition.

The MS results alone were far from convincing; however, if enzyme were unfolded when bound to the aggregate, it would likely be more susceptible to protease digestion than free, uninhibited enzyme (Figure 16). We had considered this experiment in the past, but we were concerned that the protease itself would be inhibited by the aggregates. Since then, new results have shown that the off rate from the aggregate is typically imperceptible within the timescale of most experiments and that it is possible to simply saturate aggregates with additional protein.[39, 42] Upon assaying the sensitivity of aggregate-bound β-lactamase to trypsin digestion, we saw a dramatic effect: degradation of aggregate-bound enzyme was often apparent after 15 minutes, whereas free enzyme was stable for several hours (Figure 17). β-lactamase in the absence of trypsin was unaffected by the presence of aggregates, showing that the aggregates themselves were not reducing enzyme signal. This was true for several aggregators: rottlerin, Congo Red, Eriochrome Blue Black B, nicardipine, and L-755,507 (Figure 17).

Combined with the MS results, these tryptic digests strongly indicated that the enzyme is unfolded when bound to the aggregate. But how could this be reconciled with

past experiments that had been performed, which seemed to indicate that denaturation was not the mechanism of inhibition? This led us to reconsider these previous experiments. First, why didn't additional denaturants increase enzyme inhibition by aggregates? Upon reflection, the aggregates themselves are probably solubilized by denaturants, thus reducing their concentration and potency. Second, why does GFP retain fluorescence when bound to an aggregate? GFP, we've learned, is an unusually stable enzyme, which can retain fluorescence even if it is partly unfolded.[60] Alternatively, perhaps it was not the fluorescence of the immediately bound GFP that was observed, but rather nearby GFP. Third, why wasn't a destabilized enzyme more sensitive to inhibition compared to wild type enzyme? There are several potential explanations to this question, and each illuminates that there is still far more that we do not understand about the mechanism of aggregate-based inhibition. Possibly destabilized enzyme is more sensitive to inhibition that wild-type enzyme, but the binding constants for each are both so low that the effect is imperceptible at higher concentrations such as the IC_{50}. Another explanation may be that binding to the aggregate is the rate limiting step, and that wild type or destabilized enzyme are equally likely to be bound.

Finally, how do we reconcile that we can rapidly recover enzyme activity simply by the addition of detergent? We propose that the enzyme is not completely denatured, rather only partly unfolded: enough so that its solvent accessibility and protease sensitivity has increased, but that it can easily return to its native conformation once released from the aggregate. To return to our three models (Figure 13), we have ruled out reduced dynamics and physical sequestration, as both would have resulted in reduced solvent accessibility (less deuterium exchange) and protection from digestion by a

protease. Partial unfolding appears to be the most likely mechanism – deuterium exchange would increase, as would proteolytic digestion, but the enzyme would not be so denatured that it could not easily return to its native conformation or that GFP would not retain fluorescence.

We should re-emphasize that we have not tried to draw sequence specific conclusions about the mechanism of action, although it would have been ideal to do so using the MS data. There may be peptides that appear to exchange more than others, but our margin of error is far too large to draw such conclusions. The only conclusion that we have taken from our HDX MS results is that there appears to be greater deuterium exchange in the presence of aggregates, which is consistent with an unfolding mechanism. Our time scale was also insufficient to observe the exchange rates of surface residues. Regardless, combined with the evidence that not one, but several aggregators dramatically increase an enzyme's susceptibility to proteolytic digestion, we conclude that aggregates may inhibit enzymes by partial unfolding. Although still rudimentary, this mechanism broadens our understanding of the pervasive phenomenon of nonspecific aggregate-based inhibition.

Experimental Section

Materials. AmpC β-lactamase was expressed and purified as previously described.[37] Rottlerin, Congo Red, Eriochrome Blue Black B, nicardipine, trypsin from porcine pancreas and trifluoroacetic acid were purchased from Sigma-Aldrich. L-755,507 was purchased from Tocris. Immobilized pepsin was purchased from Pierce.

HDX MS Sample Preparation. AmpC β-lactamase (215 μM stock in 50 mM KPi, pH 7.0) was delivered to the deuterated solvent (50 mM MOPS buffer in D_2O, pH 7.0) to a final concentration of 16 μM for uninhibited enzyme or 0.16 μM for samples containing the inhibitor rottlerin. The total volume of samples without rottlerin (including the undeuterated and 100% deuterated controls) was 10 μL, whereas the total volume for each sample with rottlerin was 1 mL. The concentration of rottlerin in these samples was 100 μM (0.5 % DMSO) delivered from a 20 mM stock in DMSO. The difference in β-lactamase concentration and total volume was to accommodate for the limited solubility of rottlerin. Deuterium exchange was not increased in samples containing 0.5 % DMSO (no aggregates). After incubation in the deuterated solvent for 5 minutes, 1, 2, 4 or 8 hours, the samples containing rottlerin were centrifuged for five minutes at 16,000 x g to pull down and concentrate aggregate-enzyme complexes and the top 990 μL were discarded. The bottom 10 μL were considered the pellet and were treated identically to the 10 μL samples without rottlerin from this point on.

To disrupt the aggregates, 1 μL of 3 % Triton X-100 in 50 mM deuterated MOPS buffer was added to every sample and the samples were mixed vigorously by pipetting. Immediately after, the samples were quenched with 90 μL of ice cold 0.1% TFA in water, pH 2.5. The samples were mixed and transferred to 100 μL of immobilized pepsin beads. Samples were kept on ice with occasional mixing for 5 minutes and then the pepsin beads were sedimented by centrifugation for 1 minute at 16,000 x g at 4 °C. The supernatant was collected and frozen with liquid nitrogen. Samples were kept at -80 °C until analysis.

Mass Spectrometry. The mass spectrometry data was acquired on an ABI QSTAR Pulsar hybrid LC/MS/MS system (Applied Biosystems). The mass spectrometer was equipped with the optional ABI MicroIonSpray source. Chromatography was performed using an Eldex MicroPro pump equipped with an in house splitter capable of producing column flow rates of 0.3 to 1.0 μL per minute at pump flow rates of 50-80 μL per minute. A Phenomenex Onyx monolithic column (0.100 x 150 mm) was used for all experiments. Samples were injected manually using a Valco C2-1006 injection valve with a 1.0 μL loop. Solvent A was 0.1 % formic acid in water and solvent B was 0.1 % formic acid in acetonitrile.

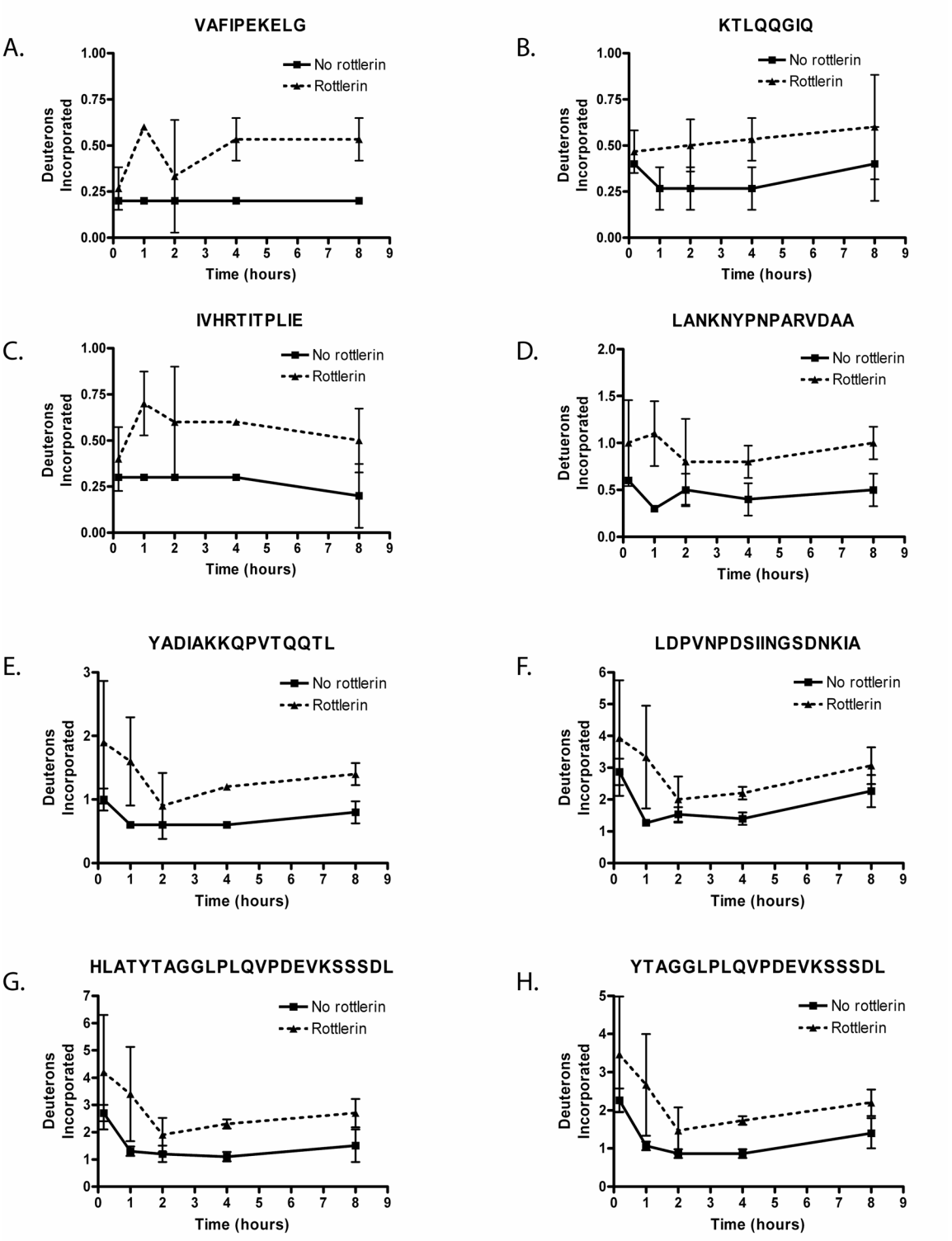
Peptide Identification. The identities of the peptides formed by digestion with pepsin were determined by LC/MS/MS analysis using the following conditions: column flow rate was 0.4 μL per minute and a gradient was run from 3% solvent B to 50 % solvent B in 35 minutes. The mass spectrometer was run using the standard IDA acquisition method. The data obtained was searched against a data base consisting only of the AmpC sequence using Protein Prospector.

Analysis of Deuterium Incorporation. For analysis of deuterated peptide samples, the same chromatography system was used. To minimize back exchange, the column flow rate was raised to 1.0 μL per minute and the gradient used was from 0 % solvent B to 70 % solvent B in 5 minutes. Total analysis time was 10-12 minutes per sample. Furthermore, the injector, injection syringe, sample loop, column and all transfer lines were maintained in an ice bath. Data was collected in MS mode only. All data analysis was performed with the standard Analyst 1.1 software native to the QSTAR. The average number of deuterons incorporated for each peptide was determined from the mass

spectra. The centroid of the undeuterated control was subtracted from the centroid of the isotopic peak cluster for each deuterated sample. Measurements were not corrected for back-exchange loss. Solvent accessible surface area was calculated using Getarea (version 1.0 beta) available at http://curie.utmb.edu/getarea.html, using a radius of 1.4 Å and default atomic radii and atomic solvent parameters.[58] The average percent accessibility was calculated as the average of the ratios of each side-chain surface area to the "random coil" value.

Tryptic Digests and Gel Electrophoresis. A sample of AmpC β-lactamase (0.5 μM) was incubated in 50 mM KPi, pH 7.0 for 1 hour with or without each aggregating inhibitor: 100 μM rottlerin, 250 μM Congo Red, 250 μM Eriochrome Blue Black B, 250 μM Nicardipine, or 100 μM L-755,507 (with a final concentration of 1% DMSO in all samples, with or without inhibitor). Samples of trypsin alone (0.01 or 0.025 mg/mL) were prepared with or without each of the inhibitors and incubated for 1 hour at room temperature in 50 mM KPi, pH 7.0. Tryptic digests of β-lactamase were prepared by first adding 0.5 μM β-lactamase to a solution with or without each inhibitor. After 5 minutes, trypsin was added to the solution and the digests were incubated at room temperature for 0 minutes, 15 minutes, 30 minutes, 1 hour, or 4 hours. The total volume of all samples was 25 μL. To prepare the samples for SDS-PAGE, 5 μL of loading buffer (containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol by volume, and 5 mM DTT) was added to each sample and the samples were boiled for 2 minutes. Samples were mixed by inversion and 13 μL was added to each well. After SDS-PAGE, protein bands were detected by silver staining.

Supplemental Figure S1. Deuterium incorporation plots for the eight additional fragments from the mass spectrum of β-lactamase in the presence (solid line) or absence (dotted line) of the aggregator rottlerin. (A) the peptide containing residues 323-332 (monoisotopic m/z = 551.8, +2 charge state), (B) residues 243-250 (monoisotopic m/z = 458.2, +2 charge state), (C) residues 8-18 (monoisotopic m/z = 431.2, +3 charge state), (D) residues 336-350 (monoisotopic m/z = 538.6, +3 charge state), (E) residues 42-56 (monoisotopic m/z = 568.6, +3 charge state), (F) residues 271-289 (monoisotopic m/z = 1091.0, +2 charge state), (G) residues 105-128 (monoisotopic m/z = 833.4, +3 charge state), and (H) residues 109-128 (monoisotopic m/z = 1038.5, +2 charge state).


A.
VAFIPEKELG
No rottlerin
Rottlerin
Deuterons Incorporated
Time (hours)
B.
KTLQQGIQ
No rottlerin
Rottlerin
Deuterons Incorporated
Time (hours)
C.
IVHRTITPLIE
No rottlerin
Rottlerin
Deuterons Incorporated
Time (hours)
D.
LANKNYPNPARVDAA
No rottlerin
Rottlerin
Detuerons Incorporated
Time (hours)
E.
YADIAKKQPVTQQTL
No rottlerin
Rottlerin
Deuterons Incorporated
Time (hours)
F.
LDPVNPDSIINGSDNKIA
No rottlerin
Rottlerin
Deuterons Incorporated
Time (hours)
G.
HLATYTAGGLPLQVPDEVKSSSDL
No rottlerin
Rottlerin
Deuterons Incorporated
Time (hours)
H.
YTAGGLPLQVPDEVKSSSDL
No rottlerin
Rottlerin
Deuterons Incorporated
Time (hours)

Acknowledgement. This work was supported by NIH grant GM71630 (to BKS) and NIH grant NCRR BRTP 01614 from the Biomedical Research Technology Program of the National Center for Research Resources (to A.L.B., director of the Bio-Organic Biomedical Mass Spectrometry Resource at UCSF). KEC was partly supported by a fellowship in the field of Pharmaceutics awarded by the PhRMA Foundation. We thank Dr. A. Falick at the University of California, Berkeley for helpful discussions. We also thank A. McReynolds and D. Teotico for β-lactamase purification, and M. Mysinger and P. Kolb for solvent accessibility calculations. We also thank D. Teotico for reading this manuscript.

Future Directions

The prevalence of aggregate-based inhibition, particularly in high-throughput screening and drug discovery, has gained it wide notoriety in the pharmaceutical industry. Although the detergent screen developed by Brian Feng is being successfully employed to reduce the amount of time and energy spent on false hits, exceptions and weaknesses of the screen are becoming apparent. Some aggregators appear to be less sensitive to detergent and so they are not being identified. Also, detergent is incompatible with some assays, particularly cell-based techniques. To develop better predictive tools and screens for aggregation, it is necessary both to expand our basic understanding of aggregation as a phenomenon, as well as to continue exploring aggregate behavior in the contexts where these molecules are used.

Expanding our fundamental understanding of small molecule aggregation

We now believe that at least some aggregates lead to enzyme inhibition by small scale enzyme denaturation. At this point it is unclear whether this mechanism holds true for all enzymes, but it is tempting to believe that there is a unifying mechanism. Similarly, different aggregates may operate by different mechanisms. Half of the aggregates we tested for increased sensitivity to proteolysis did not exhibit this behavior. We may not have had the right conditions to see this effect, but it is possible that not all aggregates are the same.

One of the early indications that there might be different types of aggregators was the different size distributions observed by flow cytometry. Most of the molecules that we tested formed aggregates around 100 nm in diameter. Unfortunately, that size was below the accurate limits of the flow cytometer and we were unable to carry our further analysis. Future developments may have lowered the limits to include that size range, in which case it would be intriguing to determine whether these smaller aggregates share the characteristics we determined about the larger particles (e.g. densely packed solid structures, proportionate stoichiometry).

In addition to studying whether there are different subclasses of aggregates, another remaining question is to determine how aggregates are assembled. We believe that at least some aggregates are densely-packed solid structures. How are the monomers organized within the aggregate? Given the diverse molecular features that are found amongst aggregating molecules, it is not apparent how they might be arranged. By eye, large conjugated ring systems combined with decorative hydrophilic functional groups seem to be a common characteristic. The most probable organization might involve stacking of these ring systems while exposing the polar groups to solvent. It might be possible to model such organization by computational methods, optimizing favorable contacts between small molecules within a constrained volume. Additionally, there may be techniques within colloid science that could illuminate aggregate structure. Comparisons to structures like liquid crystals or hydrophobic-driven organic colloids may provide clues to aggregate structure.[35, 61-63]

On a related note, why do proteins bind to the aggregate surface? Protein sticking is not a new problem and detergent is commonly used to prevent enzyme from adhering

to the walls of tubes or cuvettes. It should not be surprising that enzyme binds to small molecule aggregates, but on the other hand, colloids and micelles are not known to sequester or inhibit enzyme. What contacts are being made between the aggregate and enzyme that lead to such a potent interaction? There are techniques used to study protein behavior at surfaces, such as changes in surface density or pressure, which might be useful models.[64, 65]

Further implications of aggregate-based inhibition in drug discovery and basic research

Despite repeated attempts, we still do not know whether aggregates affect membrane-bound proteins such as receptors, transporters, and ion channels. This is a longstanding question that we have attempted to answer with multiple collaborators, including investigations of the ion channels acetylcholine receptor and P2X2, GPCRs, and organic anion transporters. The results were confounding. In each case, some aggregates appeared to have an effect, but others didn't. When it was possible to perform the experiments at different concentrations, the dose-dependency often defied explanation. Sometimes inhibition would decrease as the concentration of the aggregating molecule increased. In other cases, we would see inhibition far below where we expected the aggregates to begin forming. High-throughput assays may be the most effective technique for further addressing the question of whether aggregates inhibit membrane-bound proteins. A larger quantity of data will make it easier to determine effects and separate out biological noise.

It will also be interesting to determine whether aggregates could persist *in vivo* with simulated intestinal solutions and animal models. At this point, it seems unlikely

that they would remain for long durations, but it is possible that there could be local concentrations of small molecule high enough to sustain aggregates prior to systemic distribution. If delivered orally, it is possible that aggregates could form in the gut, as Frenkel *et al*. proposed, and perhaps the formation of particles contributes to how the small molecule is absorbed.

There are questions that remain unanswered about the relatively simple *in vitro* assays. Aggregation appears to be surprisingly condition dependent. It has been difficult to develop computational methods for predicting aggregation and this has partly been attributed to the concentration dependence of aggregate formation. The problem is even more insidious though, as aggregation also varies with buffer identity, pH, DMSO concentration, and the concentration and identity of the enzyme target. This variability has become clear as the detergent screen has been employed in several high-throughput screens. From this emerges the question of how to categorize small molecules once they are found to aggregate. If a molecule aggregates under some conditions, but not others, it seems a waste to throw it out of screening libraries, especially considering that aggregation does not preclude specific activity (as evidenced by the known drugs that aggregate *in vitro*). In short, the detergent screen is far from fool proof. Further understanding aggregate behavior in varied environments and investigating whether there are subclasses of aggregators may be useful in understanding why we see such differences.

References

1. Broach, J. R.; Thorner, J., High-throughput screening for drug discovery. *Nature* **1996,** 384, 14-16.

2. Kenny, B. A.; Bushfield, M.; Parry-Smith, D. J.; Fogarty, S.; Treherne, J. M., The application of high-throughput screening to novel lead discovery. *Prog Drug Res* **1998,** 51, 245-269.

3. Knowles, J.; Gromo, G., A guide to drug discovery: Target selection in drug discovery. *Nat Rev Drug Discov.* **2003,** 2, 63-69.

4. Gershell, L. J.; Atkins, J. H., A brief history of novel drug discovery techniques. *Nat Rev Drug Discov.* **2003,** 2, 321-327.

5. Oprea, T. I.; Davis, A. M.; Teague, S. J.; Leeson, P. D., Is there a difference between leads and drugs? A historical perpective. *J Chem Inf Comput Sci* **2001,** 41, 1308-1315.

6. Schmid, E. F.; Smith, D. A., Is pharmaceutical R&D just a game of chance or can strategy make a difference? *DDT* **2004,** 9, 18-26.

7. Brown, D.; Superti-Furga, G., Rediscovering the sweet spot in drug discovery. *DDT* **2003,** 8, 1067-1077.

8. DiMasi, J. A.; Hansen, R. W.; Grabowski, H. G., The price of innovation: New estimates of drug development costs. *J Health Econ* **2003,** 22, 151-185.

9. Sams-Dodd, F., Target-based drug discovery: is something wrong? *DDT* **2005,** 10, 139-147.

10. Walmsley, R.; Tweats, D., Reducing late stage attrition with early high-throughput genotoxicity screening. *Drug Plus International* **2006**.

11. Rishton, G. M., Nonleadlikeness and leadlikeness in biochemical screening. *Drug Discov. Today* **2003,** 8, (2), 86-96.

12. 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.; Vvon 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.

13. Walters, W. P.; Ajay; Murcko, M. A., Recognizing molecules with drug-like properties. *Curr. Opin. Chem. Biol.* **1999,** 3, (4), 384-387.

14. Hajduk, P. J.; Huth, J. R.; Fesik, S. W., Druggability indices for protein targets derived from NMR-based screening data. *J. Med. Chem.* **2005,** 48, 2518-25.

15. Rishton, G. M., Reactive compounds and *in vitro* false positives in HTS. *DDT* **1997,** 2, (9), 382-384.

16. 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, (8), 1712-22.

17. Feng, B.; Simeonov, A.; Jadhav, A.; Babaoglu, K.; Inglese, J.; Shoichet, B.; Austin, C., A high-throughput screen for aggregation-based inhibition in a large compound library. *J. Med. Chem.* **2007,** 50, (10), 2385-90.

18. Feng, B. Y., Shoichet, B.K., Small-molecule aggregates inhibit amyloid polymerization. *Nature Chemical Biology* **2008,** 4, 197-199.

19. McGovern, S. L.; Shoichet, B. K., Kinase inhibitors: not just for kinases anymore. *J. Med. Chem.* **2003,** 46, 1478-1483.

20. Seidler, J.; McGovern, S. L.; Doman, T.; Shoichet, B. K., Identification and prediction of promiscuous aggregating inhibitors among known drugs. *J. Med. Chem.* **2003,** 46, 4477-4486.

21. Frenkel, Y. V.; Clark, A. D. J.; Das, K.; Wang Y.-H.; Lewi, P. J.; Janssen, P. A. J.; Arnold, E., Concentration and pH dependent aggregation of hydrophobic drug molecules and relevance to oral bioavailability. *J. Med. Chem.* **2005,** 48, 1974-1983.

22. Fasano, M., Curry, S., Terreno, E., Galliano, M., Fanali, G., Narciso, P., Notari, S., and P. Ascenzi, The extraordinary ligand binding properties of human serum albumin. *IUBMB Life* **2005,** 57, 787-796.

23. Feng, B. Y.; Shelat, A.; Doman, T. N.; Guy, R. K.; Shoichet, B. K., High-throughput assays for promiscuous inhibitors. *Nature Chemical Biology* **2005,** 1, (3), 146 - 148.

24. DeWitte, R. S., Avoiding physiochemical artifacts in early ADME-Tox experiments. *Drug Discov. Today* **2006,** 11, 855-859.

25. Keseru, G., Makara, G., Hit discovery and hit-to-lead approaches. *Drug Discov. Today* **2006,** 11, 741-748.

26. Shoichet, B. K., Screening in a spirit haunted world. *Drug Discov. Today* **2006,** 11, (13-14), 607-615.

27. Walters, W. P.; Namchuk, M., Designing screens: how to make your hits a hit. *Nat Rev Drug Discov.* **2003,** 2, (4), 259-66.

28. Davies, S., Reddy, H., Caviano, M., Cohen, P., Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal* **2000,** 351, 95-105.

29. Liu, H.; Wang, Z.; Regni, C.; Zou, X.; Tipton, P. A., Detailed kinetic studies of an aggregating inhibitor; Inhibition of phosphomannomutase/phosphoglucomutase by Disperse Blue 56. *Biochemistry* **2004,** 27, 8662-8669.

30. Lingameneni, R., Vysotskaya, T., Duch, D., Hemmings, H., Inhibition of voltage-dependent sodium channels by Ro 31-8220, a "specific" protein kinase inhibitor. *FEBS Lett.* **2000,** 473, 265-268.

31. 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 Deliv Rev* **1997,** 23, 3-25.

32. Reddie, K., Roberts, D., Dore, T., Inhibition of kinesin motor proteins by adociasulfate-2. *J. Med. Chem.* **2006,** 49, 4857-4860.

33. Turk, B., Targetting proteases: successes, failures and future prospects. *Nat Rev Drug Discov.* **2006,** 5, 785-799.

34. McGovern, S. L.; Helfand, B. T.; Feng, B. Y.; Shoichet, B. K., A specific mechanism of nonspecific inhibition. *J. Med. Chem.* **2003,** 46, (20), 4265-4272.

35. Vieira, D., L. Pacheco, and A. Carmona-Ribeiro, Assembly of a model hydrophobic drug into cationic bilayer fragments. *Journal of Colloid and Interface Science* **2006,** 293, 240-247.

36. Bertucci, C., and E. Domenici, Reversible and covalent binding of drugs to human serum albumin: Methodological approaches and physiological relevence. *Current Medicinal Chemistry* **2002,** 9, 1463-1481.

37. Weston, G. S.; Blazquez, J.; Baquero, F.; Shoichet, B. K., Structure-based enhancement of boronic acid-based inhibitors of AmpC beta-lactamase. *J. Med. Chem.* **1998,** 41, (23), 4577-4586.

38. Ryan, A. J.; Gray, N. M.; Lowe, P. N.; Chung, C. W., Effect of detergent on "promiscuous" inhibitors. *J. Med. Chem.* **2003**, (46), 3448-3451.

39. Shoichet, B. K., Interpreting steep dose-response curves in early inhibitor discovery. *J. Med. Chem.* **2006,** 49, (25), 7274-7277.

40. Hann, M. M., Oprea, T. I., Pursuing the leadlikeness concept in pharmaceutical research. *Curr. Opin. Chem. Biol.* **2004,** 8, 255-263.

41. Veber, D. F.; Johnson, S. R.; Cheng, H. Y.; Smith, B. R.; Ward, K. W.; Kopple, K. D., Molecular properties that influence the oral bioavailability of drug candidates. *J. Med. Chem.* **2002,** 45, 2615-2623.

42. Coan, K. E. D.; Shoichet, B. K., Stability and equilibria of promiscuous aggregates in high protein milieus. *Molecular Biosystems* **2007,** 3, (3), 208-213.

43. Feng, B. Y.; Shoichet, B. K., A detergent-based assay for the detection of promiscuous inhibitors. *Nature Protocols* **2006,** 1, (2), 550-553.

44. Giannetti, A. M., Koch, B.D., Browner, M.F., Surface plasmon resonance based assay for the detection and characterization of promiscuous inhibitors. *J Med Chem* **2008,** 51, (3), 574-580.

45. http://www.bdbiosciences.com/discovery_labware/products/display_product.php?keyID=242

46. Tanford, C., Theory of micelle formation in aqueous solutions. *J. Phys. Chem.* **1974,** 78, (24), 2469.

47. Mukerjee, P.; Mysels, K. J., Critical micelle concentrations of aqueous surfactant systems. *Nat Stand Ref Data Ser, Nat Bur Stand* **1971,** 36, 1-227.

48. Smith, D.; Deng, Y.; Zhang, Z., Probing the non-covalent structure of proteins by amide hydrogen exchange and mass spectrometry. *J. Mass Spectrom.* **1997,** 32, 135-146.

49. Zhang, Z.; Smith, D. L., Determination of amide hydrogen exchange by mass spectrometry: a new tool for protein structure elucidation. *Protein Sci* **1993,** 2, 522-531.

50. Englander, S. W.; Sosnick, T. R.; Englander, J. J.; Mayne, L., Mechanisms and uses of hydrogen exchange. *Curr. Opin. Chem. Biol.* **1996,** 6, 18-23.

51. Mandell, J. M.; Falick, A. M.; Komives, E. A., Identification of protein-protein interfaces by decreased amide proton solvent accessibility. *Proc Natl Acad Sci U S A* **1998,** 95, 14705-14710.

52. Hoofnagle, A. N.; Resing, K. A.; Goldsmith, E. J.; Ahn, N. G., Changes in protein conformational mobility upon activation of extracellular regulated protein kinase-2 as detected by hydrogen exchange. *Proc Natl Acad Sci U S A* **2001,** 98, 956-961.

53. Yan, X.; Watson, J.; Ho, P. S.; Deinzer, M. L., Mass spectrometric approaches using electrospray ionization charge states and hydrogen-deuterium exchange for determining protein structures and their conformational changes *Mol and Cell Proteomics* **2004,** 3, (1), 10-23.

54. Wales, T. E.; Engen, J. R., Hydrogen exchange mass spectrometry for the analysis of protein dynamics. *Mass Spectrometry Rev.* **2006,** 25, 158-170.

55. Burlingame, A. L.; Boyd, R. K.; Gaskell, S. J., Mass Spectrometry. *Anal. Chem.* **1996,** 68, 634R-683R.

56. Mandell, J. M.; Falick, A. M.; Komives, E. A., Measurement of amide hydrogen exchange by MALDI-TOF mass spectrometry. *Anal. Chem.* **1998,** 70, 3987-3995.

57. Powers, R. A.; Caselli, E.; Focia, P. J.; Prati, F.; Shoichet, B. K., Structures of ceftazidine and its transition-state analogue in complex with AmpC beta-lactamase: implications for resistance mutations and inhibitor design. *Biochemistry* **2001,** 40, (31), 9207-9214.

58. Fraczkiewicz, R.; Braun, W., Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *J Comput Chem* **1998,** 19, 319-333.

59. Coan, K. E. D.; Shoichet, B. K., Stoichiometry and physical chemistry of promiscuous aggregate-based inhibitors. *J Am Chem Soc* **2008,** 130, (29), 9606-9612.

60. Alkaabi, K. M.; Yafea, A.; Ashraf, S. S., Effect of pH on thermal- and chemical-induced denaturation of GFP. *Applied Biochemistry and Biotechnology* **2005,** 126, (2), 149-156.

61. Lydon, Chromonic mesophases. *Current Opinion in Colloid and interface science* **2004,** 8, 480-490.

62. Beyaz, O. W.-S.; Reddy, V., Ionic liquids as modulators of the critical micelle concentration of sodium dodecyl sulfate. *Colloids and Surfaces, B* **2004,** 35, (2), 119-124.

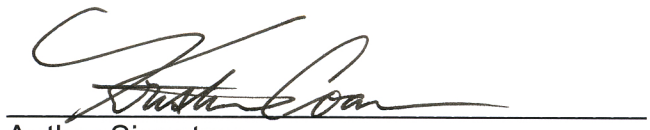
63. Skowronek, M.; Stopa, B.; Konieczny, L.; Rybarska, J.; Piekarska, B.; Szneler, E.; Bakalarski, G.; Roterman, I., Self-assembly of Congo Red - A theoretical and experimental approach to identify its supramolecular organization in water and salt solutions. *Biopolymers* **1998,** 46, 267-281.

64. Baszkin, A., Molecular recognition on the supported and on the air/water interface-spread protein monolayers. *Adv Colloid Interface Sci* **2006,** 128-130, 111-120.

65. Roach, P.; Farrar, D.; Perry, C. C., Surface tailoring for controlled protein adsorption: Effect of topography at the nanometer scale and chemistry. *JACS* **2006,** 128, 3939-3945.

It is the policy of the University to encourage the distribution of all theses and dissertations. Copies of all UCSF theses and dissertations will be routed to the library via the Graduate Division. The library will make all theses and dissertations accessible to the public and will preserve these to the best of their abilities, in perpetuity.

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis or dissertation to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.



Author Signature

Dec 11, 2008

Date