

# Stability and equilibria of promiscuous aggregates in high protein milieus

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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 millilitre 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.<sup>1,2</sup> 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.<sup>2–7</sup> Promiscuous aggregators have also been found among commonly used biological reagents, such as kinase inhibitors, Lipinski-compliant small molecules, and drugs.<sup>8–15</sup> 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.<sup>16,17</sup> 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.<sup>18</sup> 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 millilitre concentrations of BSA prior to the addition of the aggregating inhibitor and monitored enzyme.<sup>2</sup> 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,<sup>19,20</sup> 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  $\beta$ -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

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chemically reactive, irreversible  $\beta$ -lactamase inhibitor that probes the accessibility of the  $\beta$ -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  $\beta$ -lactamase was expressed and purified as described.<sup>21</sup> 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.

### $\beta$ -Lactamase assays

$\beta$ -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  $\beta$ -lactamase were incubated for five minutes and the reaction was initiated by the addition of 200  $\mu$ M nitrocefin substrate. Change in absorbance was monitored at 482 nm for 100 seconds. To determine preventative effects, 0.1 mg mL<sup>-1</sup> BSA, lysozyme, or trypsin was added to the buffer before the addition of inhibitor or  $\beta$ -lactamase. For reversal experiments, 1 mg mL<sup>-1</sup> BSA, lysozyme, or trypsin was added after a five minute incubation of  $\beta$ -lactamase with the inhibitor and the reaction was initiated by substrate immediately thereafter. In the case of TIPT, 0.1 and 10 mg mL<sup>-1</sup> 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  $\mu$ M of this substrate was used and absorbance at 405 nm was monitored. When investigating covalent inhibition by moxalactam, TIPT and  $\beta$ -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.<sup>16</sup>

### 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<sup>-1</sup> BSA, lysozyme, or trypsin in the buffer before the addition of the inhibitor. The effect of 0.1 mg mL<sup>-1</sup> 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  $\beta$ -lactamase, aggregates were incubated first with 1 nM  $\beta$ -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 Gentest<sup>®</sup> 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  $\mu$ L 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<sup>-1</sup> 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  $\mu$ L 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<sub>50</sub> values. In the absence of competing protein,  $\beta$ -lactamase activity was consistently inhibited at least eighty percent by the aggregates. Consistent with prior studies,<sup>2</sup> when 0.1 mg mL<sup>-1</sup> BSA, lysozyme, or trypsin was added to the solution before  $\beta$ -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<sub>50</sub>.

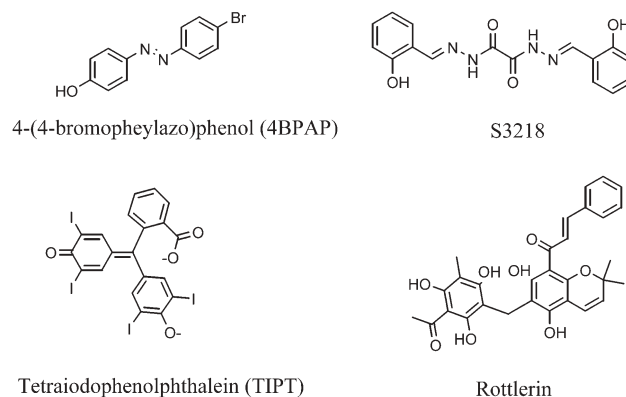
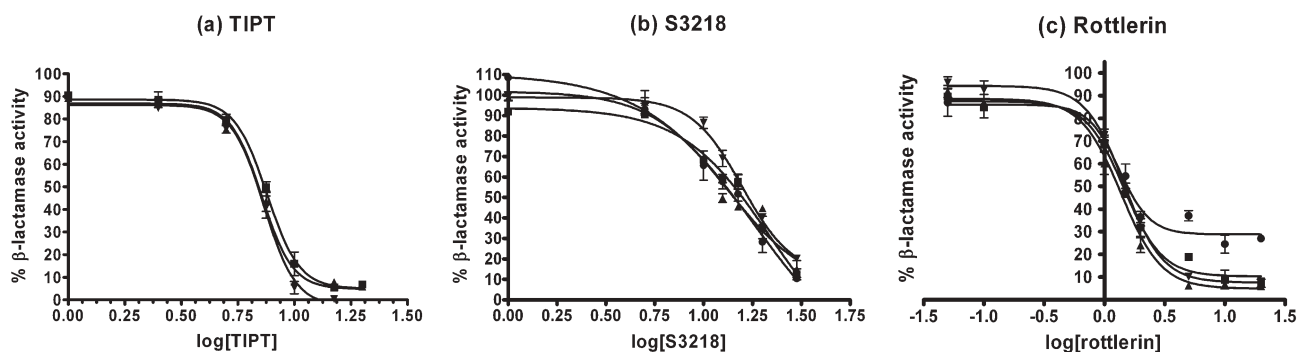


Fig. 1 Aggregators used in this study.

**Table 1**  $\beta$ -Lactamase inhibition and aggregate formation after pre-incubation with BSA, lysozyme, and trypsin

Aggregate	Conc. ( $\mu\text{M}$ )	IC <sub>50</sub>	% $\beta$ -Lactamase Activity		DLS Intensity (kcps)		
			No Protein	0.1 mg mL <sup>-1</sup> Protein	No Protein	0.1 mg mL <sup>-1</sup> Protein	Protein only
<b>Protein: Bovine Serum Albumin</b>							
TIPT	20	5	8	91	5952	3148	94
S3218 <sup>a</sup>	30	17	12	101	2910	3710	14
4BPAP	20	9	2	90	1749	2360	191
Rottlerin	5	1.5	14	89	N.M. <sup>b</sup>	N.M.	N.M.
<b>Protein: Lysozyme</b>							
TIPT	20	5	7	75	5952	5677	225
S3218 <sup>a</sup>	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.
<b>Protein: Trypsin</b>							
TIPT	20	5	6	38	5952	5660	33
S3218 <sup>a</sup>	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.

<sup>a</sup> S3218 light scattering was measured at 50% laser power. <sup>b</sup> Not measured. Rottlerin does not form particles by DLS below 10  $\mu\text{M}$ .

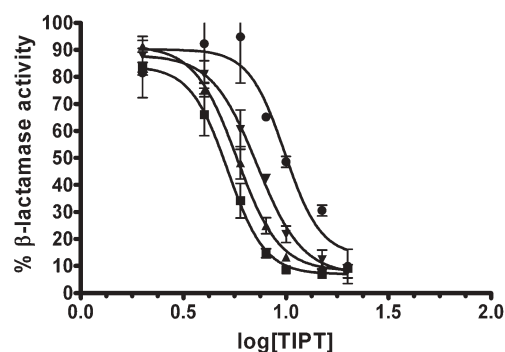


**Fig. 2**  $\beta$ -Lactamase inhibition by (a) TIPT, (b) S3218, and (c) rottlirin after the addition of competing proteins. Competing proteins were added after incubation of aggregate and  $\beta$ -lactamase: no protein ( $\blacksquare$ ), 1 mg mL<sup>-1</sup> BSA ( $\bullet$ ), 1 mg mL<sup>-1</sup> lysozyme ( $\blacktriangle$ ), and 1 mg mL<sup>-1</sup> trypsin ( $\blacktriangledown$ ). 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.

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  $\beta$ -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  $\beta$ -lactamase with aggregate. Dose-response curves were obtained for TIPT,

S3218, and rottlirin in the presence of 1 mg mL<sup>-1</sup> of BSA, lysozyme, and trypsin (Fig. 2 and 3). Rottlerin and S3218 showed no sensitivity to any of the three proteins. TIPT



**Fig. 3** BSA-dependent reversal of  $\beta$ -lactamase inhibition by TIPT with no protein ( $\blacksquare$ ), 0.1 mg mL<sup>-1</sup> BSA ( $\blacktriangle$ ), 1 mg mL<sup>-1</sup> BSA ( $\blacktriangledown$ ), and 10 mg mL<sup>-1</sup> BSA ( $\bullet$ ). TIPT and  $\beta$ -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.

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

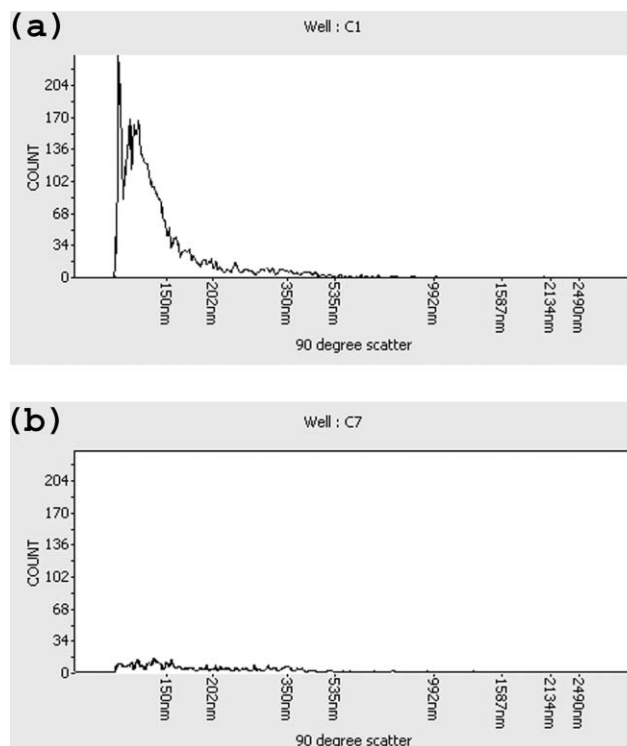
[TIPT] $\mu\text{M}$	DLS Intensity (kcps)			
	No $\beta$ -lactamase		+1 nM $\beta$ -lactamase <sup>a</sup>	
	No BSA	+0.1 mg mL <sup>-1</sup> BSA <sup>b</sup>	No BSA	+0.1 mg mL <sup>-1</sup> BSA
10	774	110	2502	703
15	4101	291	5661	2717
20	6166	2709	N.M. <sup>c</sup>	N.M.

<sup>a</sup>  $\beta$ -Lactamase only scatters 10 kcps. <sup>b</sup> BSA alone scatters 31 kcps. <sup>c</sup> Not measured.

inhibition was not reversed by lysozyme or trypsin (Fig. 2a), but was slightly reversed upon the addition of BSA (Fig. 3). We obtained dose-response curves for TIPT in the presence of no protein, 0.1 mg mL<sup>-1</sup>, 1 mg mL<sup>-1</sup>, and 10 mg mL<sup>-1</sup> BSA (Fig. 3). Although some activity was restored by BSA, this effect was modest: at 10 mg mL<sup>-1</sup> BSA, the IC<sub>50</sub> of TIPT barely doubled from 5 to 10  $\mu\text{M}$ .

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<sup>-1</sup> 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  $\mu\text{M}$  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  $\beta$ -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  $\mu\text{m}$ ) 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<sup>-1</sup> BSA to 10  $\mu\text{M}$  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.

To further explore whether there was any significant equilibrium between free and aggregate-bound enzyme, we assayed  $\beta$ -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, Case 1). Alternatively, if aggregate-bound  $\beta$ -lactamase is not irreversibly inhibited by moxalactam, it refutes both mechanisms (Fig. 5, Case 2). This would suggest that there is no measurable dynamic equilibrium between bound and free



**Fig. 4** Size distribution histograms obtained by flow cytometry for (a) 10  $\mu\text{M}$  TIPT and (b) 10  $\mu\text{M}$  TIPT + 0.1 mg mL<sup>-1</sup> BSA.

enzyme and that the enzyme active site is protected from covalent modification.  $\beta$ -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,<sup>16,22</sup>  $\beta$ -Lactamase activity was restored. The initial amount of enzyme activity restored in the presence of TIPT and moxalactam was no less than that restored by  $\beta$ -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.  $\beta$ -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  $\beta$ -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.<sup>23</sup>

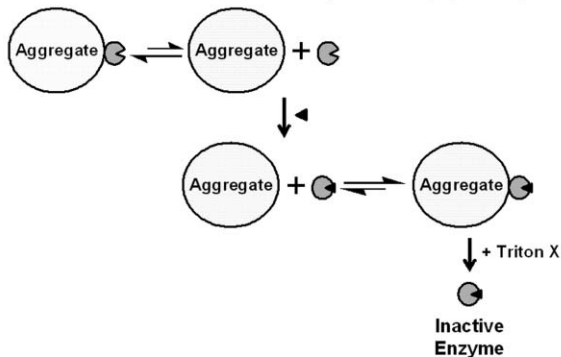
## Discussion

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.<sup>1,4,6,13</sup> These studies do not support that view. Although aggregates can be saturated by protein, they are not typically disrupted at milligram per millilitre concentrations of protein. Furthermore, once formed, the aggregate-enzyme complex is unperturbed by additional protein. Thus,

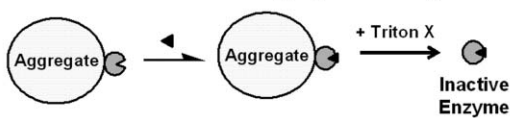
## Case 1

### Aggregate-bound enzyme is inhibited by moxalactam:

Mechanism 1: Moxalactam binds free enzyme indicating dynamic equilibrium



Mechanism 2: Moxalactam binds aggregate-bound enzyme



## Case 2

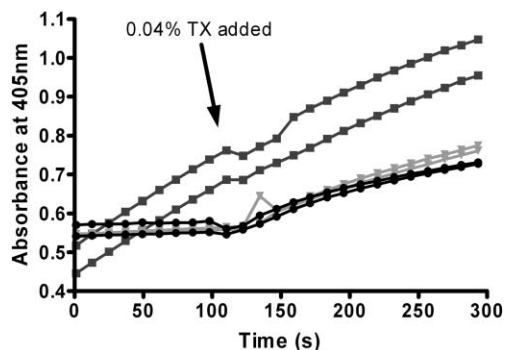
### Aggregate-bound enzyme is not inhibited by moxalactam:



There is little free enzyme and the irreversible inhibitor cannot bind aggregate-bound enzyme

● = enzyme ◀ = irreversible inhibitor: moxalactam

**Fig. 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 light grey and the irreversible inhibitor is represented by a black triangle (see key at bottom of figure).



**Fig. 6** Aggregate disruption by 0.04% Triton X-100 added during a  $\beta$ -lactamase inhibition assay containing  $\beta$ -lactamase (■),  $\beta$ -lactamase with 25  $\mu\text{M}$  TIPT (▼), and  $\beta$ -lactamase treated first with 25  $\mu\text{M}$  TIPT followed by incubation with 1  $\mu\text{M}$  moxalactam (●). Triton X-100 (TX) was added approximately 100 s after the reaction was initiated with substrate.

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.<sup>19,20</sup> 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  $\text{IC}_{50}$ 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  $\beta$ -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.<sup>16</sup> 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  $\text{mg mL}^{-1}$  BSA, TIPT  $\text{IC}_{50}$  values barely doubled and by 20  $\mu\text{M}$  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  $\beta$ -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  $\beta$ -lactamase is inaccessible to the irreversible inhibitor moxalactam, even after a 30 minute

incubation (data not shown), suggesting that  $\beta$ -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.<sup>23</sup> 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.<sup>18</sup> 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 millilitre 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

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