The staphylococcal biofilm protein Aap forms a tetrameric species as a necessary intermediate before amyloidogenesis

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The accumulation-associated protein (Aap) from Staphylococcus epidermidis is a biofilm-related protein that was found to be a critical factor for infection using a rat catheter model. The B-repeat superdomain of Aap, composed of 5–17 B-repeats, each containing a Zn$$^{2+}$$-binding G5 and a spacer subdomain, is responsible for Zn$$^{2+}$$-dependent assembly leading to accumulation of bacteria during biofilm formation. We previously demonstrated that a minimal B-repeat construct (Brpt1.5) forms an antiparallel dimer in the presence of 2–3 Zn$$^{2+}$$ ions. More recently, we have reported the presence of functional amyloid-like fibrils composed of Aap within S. epidermidis biofilms and demonstrated that a biologically relevant construct containing five and a half B-repeats (Brpt5.5) forms amyloid-like fibrils similar to those observed in the biofilm. In this study, we analyze the initial assembly events of the Brpt5.5 construct. Analytical ultracentrifugation was utilized to determine hydrodynamic parameters of reversibly associating species and to perform linked equilibrium studies. Linkage studies indicated a mechanism of Zn$$^{2+}$$-induced dimerization similar to smaller constructs; however, Brpt5.5 dimers could then undergo further Zn$$^{2+}$$-induced assembly into a previously uncharacterized tetramer. This led us to search for potential Zn$$^{2+}$$-binding sites outside of the dimer interface. We developed a Brpt5.5 mutant that was unable to form the tetramer and was concordantly incapable of amyloidogenesis. CD and dynamic light scattering indicate that a conformational transition in the tetramer species is a critical step preceding amyloidogenesis. This mechanistic model for B-repeat assembly and amyloidogenesis provides new avenues for potential therapeutic targeting of staphylococcal biofilms.

The human skin commensal, Staphylococcus epidermidis, has been referred to as the “accidental pathogen” (1). Its primary virulence factor is its apt ability to form biofilms on a variety of surfaces (2). It is this feature that has allowed S. epidermidis to take its place as a leading cause of hospital-acquired infections (3) and, specifically, as the primary culprit in device-related infections (4). Biofilms are well-organized bacterial communities that offer a high degree of mechanical and chemical resistance to its constituent cells (5). Biofilm formation begins with the attachment of bacteria to a biotic (e.g. corneocytes or collagen-coated implant) or abiotic surface (e.g. catheter or artificial joint). Following attachment, the accumulation of bacteria is mediated by protein-protein interactions and/or secretion of the extracellular polysaccharide, poly-GlcNAc. As the biofilm matures, its characteristic three-dimensional structure takes shape. Eventually, cycles of dispersal of planktonic bacteria from the biofilm occur, allowing for the cycle to begin again with establishment of biofilms and infection at locations distal to the original site (2).

One of the key determinants of biofilm formation, specifically in the context of infection, is the expression of accumulation-associated protein (Aap) (6–9). This protein is anchored to the peptidoglycan layer of the staphylococcal cell wall at its C terminus. Starting at the N terminus is a series of short A-repeats, followed by a lectin domain flanked by proteolytic cleavage sites, the B-repeat superdomain containing up to 17 B-repeats composed of Zn$$^{2+}$$-binding G5 domains and spacer regions, and, last, a highly extended proline/glycine-rich stalk region (Fig. 1) (10). Although the A-repeats and/or lectin domain are required for Aap’s role in staphylococcal attachment to a surface, removal of these regions via cleavage by SepA or other proteases is required for the accumulation of bacteria in the biofilm via B-repeat self-assembly (11, 12). Biophysical and crystallographic studies on minimal B-repeat constructs containing one and a half B-repeats (Brpt1.5; a full B-repeat containing a G5 domain and spacer region, plus a C-terminal G5 domain) have shown that B-repeats are highly extended, rich in β-sheet and random coil secondary structure, and monomeric in the absence of Zn$$^{2+}$$(9, 13–16). When Zn$$^{2+}$$ is present, Brpt1.5 dimerizes in a mostly overlapping, anti-parallel fashion with no observable change in secondary structure. In the crystal structure, a Zn$$^{2+}$$ ion is bound to each G5 domain and interacts with both protomers (Fig. 1) (15). The residues involved in Zn$$^{2+}$$ binding have been identified by crystallography and mutagenesis (15). Our laboratory has also shown that Cu$$^{2+}$$ can support assembly of B-repeats, whereas Mn$$^{2+}$$, Co$$^{2+}$$, and Ni$$^{2+}$$ can bind but do not support assembly (16).

Another important feature is that whereas the B-repeats are 89–100% identical, each Aap B-repeat exists as one of two subtypes. These two subtypes differ in eight residues in the G5 domain that swap in and out as a “cassette” of residues. These residues are located near the Zn$$^{2+}$$-binding site, dimer interface, and hydrophobic “stack” in the Brpt1.5 dimer structure. Interestingly, the variant B-repeats with the less common cassette show weaker Zn$$^{2+}$$-dependent dimerization, but higher thermal stability, compared with the more common consensus repeats in Brpt1.5 constructs (13).
Whereas the Zn$^{2+}$-dependent assembly of Brpt1.5 constructs has been well-explored, Aap is believed to require at least five B-repeats to support biofilm formation, given results observed in its S. aureus ortholog, SasG (17). Our laboratory has previously sought to characterize the Zn$^{2+}$-dependent assembly of longer, more biologically relevant constructs (namely a Brpt5.5 construct), and we identified the formation of larger, Zn$^{2+}$-induced species leading up to the formation of functional amyloid-like fibrils. The presence of amyloid fibrils in S. epidermidis biofilms was demonstrated, and we also showed that the fibrils are composed primarily of proteolytically processed Aap. These fibrils are likely to offer physical resilience to the biofilm as well as resistance to chemical insults: for example, the Brpt5.5 amyloid fibrils, but not oligomers, resist disassembly by the Zn$^{2+}$ chelator diethylenetriamine pentaacetate or by acidic pH. In contrast, the addition of diethylenetriamine pentaacetate completely inhibits Brpt5.5 assembly in solution and inhibits initial biofilm formation (but has no effect on mature, preformed biofilms) (18).

In this report, we first focus on characterizing the initial, reversible assemblies formed by Brpt5.5 in the presence of Zn$^{2+}$ using analytical ultracentrifugation. Our analyses demonstrate the formation of a Brpt5.5 dimer and tetramer, in contrast to Brpt1.5 constructs, which only form dimer (9, 13, 15, 19). By analysis of the linked equilibria between Zn$^{2+}$-binding and Zn$^{2+}$-mediated B-repeat assembly, we report the number of Zn$^{2+}$ ions bound upon dimerization and tetramerization. We utilized chemical modifications and site-directed mutagenesis to narrow down our search for residues in the tetramer interface and Zn$^{2+}$-coordination sites, resulting in the development of a tetramer-negative mutant. Native Brpt5.5 undergoes a temperature-dependent conformational change in the presence of Zn$^{2+}$ that correlates with rapid aggregation; however, the tetramer-negative mutant did not undergo this conformational change or the subsequent aggregation, suggesting that formation of the tetramer is a critical step in amyloidogenesis of tandem B-repeats. Finally, we propose models of the dimer and tetramer assembly states formed by Brpt5.5.

### Results

**Brpt5.5 exhibits monomer-dimer-tetramer equilibrium**

To initiate our investigation into the assembly of Brpt5.5, we performed sedimentation velocity analytical ultracentrifugation (AUC) experiments at increasing ZnCl$_2$ concentrations at a constant Brpt5.5 concentration (Fig. 2A and Fig. S1). In the absence of ZnCl$_2$, Brpt5.5 is monomeric at all concentrations tested (Fig. S2) with a fractional ratio of 3.33 (Table 1), indicating a highly elongated conformation (compact, globular proteins have a fractional ratio of ∼1.2–1.4 (20, 21)). We observed an increase in the sedimentation coefficient as the ZnCl$_2$ concentration was increased. As expected, based on the dimerization of shorter B-repeat constructs (9, 13, 15, 16, 19), the distribution shifted, rather than showing independent peaks locked at specific $s^*$ values that change only in intensity. The shifting or sliding of distributions upon assembly is characteristic of a reversible equilibrium exhibiting fast kinetics on the time scale.

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**Figure 1.** A, domain arrangement of Aap from S. epidermidis RP62A. The N terminus contains short repeats followed by a lectin domain. A proteolytic cleavage site allows for removal of this region during biofilm accumulation, exposing the B-repeat superdomain, a repetitive proline/glycine-rich region, and a cell wall-anchoring motif. B, a model of Brpt5.5 with each B-repeat labeled, with numbers corresponding to position in the scheme from A. C and D, structure of the Brpt1.5 dimer (with two Zn$^{2+}$ ions shown as black spheres; Protein Data Bank entry 4FUN). The model of Brpt5.5 shown in B is constructed from the superposition of multiple Brpt1.5 models, with the N-terminal half-repeat overlaying the G5 domain from each of the other B-repeats. These images were generated using PyMOL (PyMOL Molecular Graphics System, version 1.8, Schrödinger, LLC).

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**EDITORS’ PICK: An Aap tetramer intermediate is required for amyloidogenesis**

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of the sedimentation velocity experiment (22–25). Also, a shift from ~2.2 to ~6.5 S is unexpectedly large for a monomer-dimer assembly (Brp1.5 dimerization resulted in a shift from ~1.5 to ~2.7 S (9, 13)). Based on our previous observations of higher-order assembly of Brpt5.5 in the presence of Zn$^{2+}$ (18), we interpreted the sedimentation velocity results as suggesting assembly beyond a dimer. Fig. 2B shows the relationship between the weight-average sedimentation coefficient ($\hat{s}$) and the Zn$^{2+}$ concentration. At 8 mM ZnCl$_2$, there is significant loss of protein due to aggregation prior to running the experiment but no further shift in $\hat{s}$ compared with the 7 mM ZnCl$_2$ condition.

Experiments performed at 2- and 3-fold higher protein concentrations showed only a minor increase in sedimentation (Fig. S3), indicating that we are observing a true species rather than a reaction boundary between two species. Given that the 8 mM ZnCl$_2$ data essentially describe a single species (the largest soluble species before aggregation), we can estimate the frictional ratio of the terminal species at 1.44: less elongated than the monomer but still indicative of an elongated, nonglobular shape (Table 1).

To better define the species present during Zn$^{2+}$-dependent assembly, a sedimentation equilibrium AUC experiment was performed at 3 mM ZnCl$_2$, where all species should be populated to an observable degree, based on the sedimentation velocity data. A global fit was performed using nonlinear least squares analysis on data at three different protein-loading concentrations and at least three speeds. The data were best fitted by a monomer-dimer-tetramer (1-2-4) equilibrium, compared with 1-2, 1-3, 1-4, 1-2-3, and 1-3-4 (Table S1). Fig. 2C shows the raw data and species fits for the middle concentration of 1.9 $\mu$m (0.15 mg/ml) sample, with residuals shown in the upper plot. Higher and lower ZnCl$_2$ concentrations also showed a 1-2-4 equilibrium (see data in Fig. 3 as well as Fig. S4). This is the first data confirming formation of a tetramer by tandem B-repeats. A species plot derived from the experimentally fitted association constants (Fig. 2D) revealed overlapping populations of the monomer, dimer, and tetramer forms of Brpt5.5. Species plots at other Zn$^{2+}$ concentrations show a similar trend (Fig. S4).

**Figure 2. Brpt5.5 exhibits a monomer-dimer-tetramer equilibrium in the presence of Zn$^{2+}$. A, WDA of 0.50 mg/ml Brpt5.5 in the presence of increasing ZnCl$_2$ concentrations, starting at 0 mM ZnCl$_2$ at the left in purple, shifting to the right up to 8 mM ZnCl$_2$ in green. B, the weight-average sedimentation coefficient ($\hat{s}$) and weight-average frictional ratio ($f/f_0$) are from SEDFIT's $\hat{s}$ analysis. Values are not reported for Brpt5.5 WT dimer, as this species can neither be isolated nor highly enriched under these conditions. For Brpt5.5 WT tetramer and 5xH85A dimer, minor amounts of monomer and/or dimer are present; therefore, the reported values are only approximations. Data were collected at 3.50, 5.00, and 8 mM ZnCl$_2$, providing results within 0.5 S of the presented data. C, a representative sedimentation equilibrium AUC data set at 3 mM Zn$^{2+}$, 0.15 mg/ml Brpt5.5, at 13,000 rpm. This data set is part of a global fit of six or more curves (at least three protein concentrations and at least three speeds). Empty circles, raw absorbance data at 236 nm; solid gray line, best fit, with residuals shown in the top plot. Individual species are represented by lines in black (monomer), red (dimer), and blue (tetramer). D, distribution of each species based on Brpt5.5 concentration (6.5 $\mu$m = 0.50 mg/ml), calculated from the determined association constants at 3 mM Zn$^{2+}$. The x axis extends until saturation of monomer or tetramer.

**Table 1**

Hydrodynamic parameters determined by AUC in this study

| Sample | [ZnCl$_2$] | Sedimentation Coefficient | $f/f_0$ |
|--------|------------|---------------------------|---------|
| Brpt5.5 monomer | 0 | 2.20 | 3.33 |
| Brpt5.5 dimer | NA | NA | NA |
| Brpt5.5 tetramer | 8 | 7.07 | 1.44 |
| Brpt5.5 5xH85A monomer | 0 | 2.27 | 3.50 |
| Brpt5.5 5xH85A dimer | 8 | 3.97 | 2.99 |

All values were determined from data collected at 0.50 mg/ml (6.5 $\mu$m) protein at 20°C in 50 mM MOPS, pH 7.2, 50 mM NaCl (with the addition of the specified ZnCl$_2$), except for Brpt5.5 WT tetramer, where 1.50 mg/ml protein was used to ensure as much saturation of the tetramer as possible (Fig. S3). The weight-average sedimentation coefficient ($\hat{s}$) and weight-average frictional ratio ($f/f_0$) are from SEDFIT's $\hat{s}$ analysis. Values are not reported for Brpt5.5 WT dimer, as this species can neither be isolated nor highly enriched under these conditions. For Brpt5.5 WT tetramer and 5xH85A dimer, minor amounts of monomer and/or dimer are present; therefore, the reported values are only approximations. Data were collected at 20°C. NA, not applicable.
Whereas we have previously reported that there is no change in the secondary structure of Brpt1.5 upon dimerization (9), we tested for the presence of any changes that might occur in the secondary structure of Brpt5.5 upon assembly. In the presence of 5 mM ZnCl$_2$, where Brpt5.5 should exist as a mix of dimer and tetramer, there was little to no change in the secondary structure by CD (Fig. S5). This indicates that there is likely no major local conformational change throughout this reversible assembly. In the context of Brpt1.5, we infer the lack of secondary structure changes and the relatively minimal contact area between the two protomers in the crystal structures as meaning that there is little difference, structurally, between the protomers in the monomer and dimer state. The shared coordination of Zn$^{2+}$ between the two protomers in the dimer is likely the dominant stabilizing force (15).

**Analysis of linked equilibria indicates that Brpt5.5 and shorter constructs share similar assembly mechanisms**

We previously analyzed the linked equilibria between Zn$^{2+}$-binding and Zn$^{2+}$-mediated Brpt1.5 and Brpt2.5 dimerization, revealing that dimerization was linked to the binding of $\sim$2 Zn$^{2+}$ ions; this is consistent with the X-ray crystal structure of Brpt1.5, which forms an anti-parallel dimer around two Zn$^{2+}$ ions (no structural data are available for Brpt2.5) (9, 15, 19). Sedimentation equilibrium AUC experiments were performed with Brpt5.5 at 15 ZnCl$_2$ concentrations, and the dimerization and tetramer assembly constants were determined. For the dimerization association constant, $K_{12}$, the slope of the log-log Wyman plot indicated that $8.1 \pm 1.0$ Zn$^{2+}$ ions are bound upon dimerization (Fig. 3A). A comparison of the number of Zn$^{2+}$ ions bound during dimerization as a function of the number of G5 domains for Brpt5.5, Brpt2.5, and Brpt1.5 (9, 19) revealed an apparent linear trend, with a slope of $1.3 \pm 1.0$ Zn$^{2+}$ ions bound per G5 domain, consistent with the 1–2 Zn$^{2+}$ ions per G5 domain reported previously (9, 19) (Fig. 3B).

**Formation of the tetramer requires additional Zn$^{2+}$ ions**

We then produced a Wyman plot for the overall tetramerization constant, $K_{14}$ (Fig. 3C) determined in the linked equilibrium analysis, along with the stepwise dimer-tetramer assembly constant, $K_{12}$ (Fig. 3D). The slope of the $K_{14}$ Wyman plot was $24.2 \pm 1.7$, and that of the $K_{24}$ Wyman plot was $5.4 \pm 1.6$. These data indicate that whereas dimerization of Brpt5.5 requires 7–9 Zn$^{2+}$ ions, 4–7 additional Zn$^{2+}$ ions are required for the two dimeric species to assemble into a Brpt5.5 tetramer.

**Chemical modification to probe the tetramer surface interface**

To probe surface residues of Brpt5.5 required for tetramer formation, we chemically modified residues expected to be outside of the dimer interface, based on the Brpt1.5 dimer crystal structure solved (Fig. 4) (15). Because our analysis of linked equilibria suggested a similar dimerization mechanism across these B-repeat constructs, we expect this to be a useful strategy to probe Brpt5.5 tetramerization.
SITE-DIRECTED MUTAGENESIS TO DEFINE THE Zn²⁺-COORDINATION SITE IN THE TETRAMER

After determining from the Wyman plots that 4–7 additional Zn²⁺ ions are required for tetramer formation, we began searching for another Zn²⁺-binding site. Fig. 4 (inset) shows a potential Zn²⁺-binding site that contains a His (His-85) and several negatively charged residues (Asp-87, Glu-100, and Asp-122) that could coordinate a Zn²⁺ ion. These residues are present in each B-repeat spacer region (Fig. S6). This hypothesized Zn²⁺ binding is located near Tyr-126, one of the residues that decreased tetramer formation upon chemical modification (Fig. 5). We chose the histidine in position 85 of each B-repeat spacer region for further investigation, due to the importance of His-75 in dimerization (15) and the general prevalence of His residues in Zn²⁺-coordination sites (26).

To test our hypothesis that His-85 is required for Zn²⁺-dependent tetramerization, we produced a Brpt5.5 pentamutant containing a H85A mutation in each spacer region of Brpt5.5 (i.e. H85A, H213A, H341A, H469A, and H597A), which we will refer to as Brpt5.5 5xH85A for simplicity. Note that there are only five spacer regions, whereas there are six G5 domains in Brpt5.5, because the C-terminal half-repeat is a final G5 domain. The 5xH85A mutant revealed a secondary structure similar to that of the native Brpt5.5 construct by far-UV CD but showed an 8°C decrease in thermal stability, likely due to the involvement of the His-85 residues in a hydrogen-bonding network and electrostatic interactions in each spacer region (Figs. S7 and S8).

We performed a series of sedimentation velocity experiments examining Zn²⁺-dependent assembly of Brpt5.5 5xH85A (Fig. 6A). In the absence of ZnCl₂, 5xH85A was monomeric with a similar sedimentation coefficient and frictional ratio for 5xH85A as observed for WT (Table 1 and Fig. S2). Along with the previously mentioned CD results, these data suggest that the H85A mutations do not significantly disrupt local secondary structure or global conformational preferences. With increasing ZnCl₂ concentrations, 5xH85A displays an increase in the weight-average sedimentation coefficient (s̄) values up to a maximum of ~4 S compared with ~6.5 S for WT (Fig. 6B and Fig. S3). Individual distributions comparing 3.50 mM ZnCl₂ and 8 mM ZnCl₂ clearly show significant inhibition of assembly by the 5xH85A mutant (Fig. 6B, inset). Furthermore, the apparent single sigmoidal transition in the 5xH85A weight-average sedimentation coefficient data suggests that only a monomer-dimer equilibrium is present.

Equilibrium AUC experiments performed with Brpt5.5 WT and 5xH85A in the presence of Zn²⁺ revealed very little change in the dimerization constants (Fig. 6C and Table 2). However, under conditions in which the WT construct predominantly assembled into tetramer, no tetramer species was detectable for 5xH85A (Fig. 6D). Equilibrium assembly constants from global fitting of the AUC data for WT Brpt5.5 and 5xH85A are shown in Table 2. These data suggest that His-85 in the spacer region is absolutely critical for tetramer formation but is not involved in dimerization.

HIS-85-DEPENDENT Zn²⁺ COORDINATION IS REQUIRED FOR BRP5.5 AGGREGATION

Because we did not observe aggregation at high Zn²⁺ concentrations during initial characterization of the Brpt5.5 5xH85A construct, we were interested in testing the ability of Brpt5.5 5xH85A to form Zn²⁺-induced amyloid-like fibrils. With WT Brpt5.5 in the presence of 5 mM ZnCl₂, we observe a major change in the far-UV CD spectrum at ~225 nm as temperature is increased (Fig. 7A, left). Interestingly, Brpt5.5 5xH85A under the same conditions appears to simply unfold as the temperature is increased (Fig. 7A, right). The strong minimum observed near 40°C for WT is likely representative of major rearrangement or twisting of β-sheets (27) into a nucleating species on the pathway to amyloidogenesis, as the appearance of the minimum at 225 nm was immediately accompanied by heavy aggregation in the cuvette and loss of CD signal. We have previously shown that Brpt5.5 under these conditions
forms fibers that could be detected by the anti-amyloid OC antibody and amyloid- or aggregation-detecting dyes (18). Furthermore, upon cooling the sample at the end of the experiment, Brpt5.5 5xH85A refolded to its native secondary structure, whereas Brpt5.5 WT exhibited virtually complete irreversibility and failed to regain its native structure (Fig. 7A, black line).

We evaluated the ability of Brpt5.5 5xH85A to form Zn$^{2+}$-induced aggregates via two other methods as well. We monitored light scattering as ZnCl$_2$ was titrated into a cuvette of Brpt5.5 WT or Brpt5.5 5xH85A (Fig. 7B), and we observed a sigmoidal transition with a midpoint near 15–20 mM ZnCl$_2$ for WT. For Brpt5.5 5xH85A, we instead observed a very gradual transition that required higher ZnCl$_2$ concentrations for turbidity. Based on these data alone, we cannot say with certainty whether this observed turbidity is related to a very weak propensity for amyloid-like aggregation or if this is nonordered or nonspecific aggregation.

We used a third complementary technique, dynamic light scattering (DLS), to measure Brpt5.5 particle size in the presence of Zn$^{2+}$ as a function of temperature. This approach, in parallel with CD measurements, can provide us with two unique perspectives: local secondary structure changes and global aggregation. In good agreement with our CD observations (Fig. 7A), we observe aggregation by DLS of Brpt5.5 WT near 37 °C, consistent with the appearance of the 225-nm minimum and subsequent loss of signal by CD (Fig. 7C). In the case of Brpt5.5 5xH85A, there is instead a significant decrease in the hydrodynamic radius ($R_h$) which is mirrored by the unfolding to random coil observed by CD near 50°C (Fig. 7D). Due to the highly elongated nature of the B-repeats, unfolding of Brpt5.5 5xH85A to a random coil is expected to be described by a decrease in $R_h$, contrary to the unfolding of a compact, globular protein. We therefore conclude that the His-85 residue and its equivalent residues in each spacer subdomain are critically linked to Zn$^{2+}$-induced B-repeat amyloidogenesis.

Table 2

| Sample | $K_{12}$ | $K_{14}$ |
|--------|---------|---------|
| Brpt5.5 WT + 3.50 mM ZnCl$_2$ | $3.31 \times 10^3$ ($1.25 \times 10^3$ to $9.26 \times 10^3$) | $6.28 \times 10^{15}$ ($1.64 \times 10^{15}$ to $2.57 \times 10^{16}$) |
| Brpt5.5 5xH85A + 3.50 mM ZnCl$_2$ | $2.15 \times 10^5$ ($1.09 \times 10^5$ to $4.52 \times 10^5$) | |

We measured equilibrium constants and 95% confidence limits from sedimentation equilibrium experiments shown in Fig. 6 (C and D) Data were collected at 20 °C.
due to their role in the formation of an obligatory tetrameric intermediate state.

Discussion

Based on the results presented in this study, we can propose models for the Brpt5.5 dimer and tetramer assembly. Linkage equilibrium studies suggested a consistent mechanism of dimerization between minimal Brpt1.5 constructs, a Brpt2.5 construct, and Brpt5.5 presented here. The number of Zn$^{2+}$ ions bound upon dimerization was found to be a consistent 1–2 Zn$^{2+}$ ions per G5 domain. X-ray crystallography structures for Brpt1.5 (15) show an “overlapping,” anti-parallel dimer, where there are sufficient Zn$^{2+}$-dependent contacts to accommodate the appropriate number of Zn$^{2+}$ ions. We propose that Brpt5.5 assembles into a similar “overlapping” dimer (Fig. 8, middle right) as opposed to a more offset dimer (Fig. 8, middle left). This model for Brpt5.5 dimerization is also consistent with the B-repeat subtype pattern found in Aap from this strain of S. epidermidis (RP62A); Brpt5.5 contains one N-terminal variant repeat, followed by four consensus repeats. The “overlapping” model predicts that all four consensus (more assembly-competent) B-repeats can make contact, whereas the variant (less assembly-competent) B-repeat overlaps with the half-repeat cap.

Due to the number of B-repeats, one could imagine a variety of orientations or configurations for the tetramer (a dimer of dimers). In Fig. 8, we evaluate the plausibility of different configurations of each assembly state. Based on our hydrodynamic data from sedimentation velocity AUC experiments (Table 1), the frictional ratio decreases from monomer to tetramer for WT Brpt5.5. Because we cannot isolate the dimer using WT Brpt5.5, we cannot accurately estimate the frictional ratio of this species. However, using the 5xH85A mutant, which dimerizes similarly to WT, we can in fact saturate the dimer population, giving us a frictional ratio smaller than that of the monomer (i.e., slightly less elongated). The “overlapping” dimer we proposed based on linkage studies would indeed exhibit a smaller frictional ratio than the monomer, as it is essentially twice as thick in the z-direction but similar along the other two axes. Importantly, in the “offset” dimer model, there would not be enough Zn$^{2+}$-binding sites in contact to satisfy our observation of 1–2 Zn$^{2+}$ ions bound per G5 domain upon dimerization. The transition from the overlapping dimer to the tetramer is accompanied by a further decrease in the frictional ratio. If dimers attached end-to-end to form the tetramer, there would be a significant extension along the x axis, whereas the other axes would be unchanged. This would result in a much higher frictional ratio of the tetramer compared with the dimer. Another option for the tetramer is a “side-by-side” dimer of dimers. There could be several arrangements fitting this description, but each variation presented in Fig. 8 would be expected to yield a lower frictional ratio than the dimer, as was observed. A tilted or twisted side-by-side dimer of dimers could also be plausible, given that our linked equilibrium result suggests that as few as four additional Zn$^{2+}$ ions may be required for formation of the tetramer (suggesting that not all B-repeats

**Figure 7. Inhibiting tetramer formation results in weaker aggregation propensity.** A, far-UV wavelength scans at 10°C increments, from 20 to 90°C. The black spectra represent scans taken at 20°C, after the sample had been heated to 90°C. Brpt5.5 WT shows significant change in the Brpt5.5 CD signal at 40°C in the presence of Zn$^{2+}$ (specifically ~225 nm), whereas the 5xH85A mutant does not show this behavior. B examines the turbidity of Brpt5.5 WT (black circles) or 5xH85A (red circles) upon Zn$^{2+}$ additions. Data points plotted are averages from three replicate experiments, with error bars showing S.D. C and D, the $R_i$ measured by DLS (black circles) overlaid with CD data collected at a single wavelength (red circles; wavelength specified on the right y axis). DLS data points are averages from triplicate measurements at each temperature during a single experiment. DLS error bars show ± S.D., whereas CD error bars are the error reported by the instrument. The DLS data sets presented are representative of at least two experiments performed at 0.5–1 mg/ml with 3.50 mM ZnCl$_2$, whereas CD data sets shown are representative of at least two experiments performed at 0.5–1 mg/ml with 3.50 mM ZnCl$_2$ or 5 mM ZnCl$_2$.
The side-by-side models are also more reasonable than the “end-to-end” model in a biological sense, as adjacent copies of cell wall–anchored Aap extending outward from the opposing staphylococcal cell surfaces would be available to interact with neighboring Aap molecules after the initial dimerization event occurs.

Our previous demonstration of the ability of the B-repeats of Aap to form a functional amyloid in the presence of Zn\(^{2+}\) was an early indication that tandem B-repeats (longer than the Brpt1.5 construct) have the ability to assemble into higher-order oligomers (18). This study revealed that Brpt5.5 exhibits a monomer-dimer-tetramer reversible equilibrium in the presence of Zn\(^{2+}\). In fact, we found that inhibiting formation of the tetramer through mutation of predicted Zn\(^{2+}\)-binding sites also prevented a conformational change associated with amyloidogenesis. This raises the intriguing possibility that the tetrameric species acts as a nucleating species for amyloidogenesis and may represent a target for future therapeutics designed to inhibit formation of functional amyloid in S. epidermidis biofilms—a component that appears to confer a high degree of physical strength and chemical resistance to the biofilm.

**Materials and methods**

**Protein expression and purification**

Brpt5.5 WT cloning, expression, and purification was performed exactly as described previously (18). The Brpt5.5 5xH85A mutant was produced via the Agilent QuickChange II Site-Directed Mutagenesis Kit. Mutated residues include His-85, His-213, His-341, His-469, and His-597, all of which were mutated to alanine. Primers used for mutagenesis are listed in Table S2. Brpt5.5 5xH85A was purified using the same procedures as Brpt5.5 WT.

**Analytical ultracentrifugation**

A Beckman Coulter XL-1 analytical ultracentrifuge was used for AUC experiments. For sedimentation velocity experiments, two-sector epon-charcoal 1.2-cm centerpieces were used with sapphire windows. Data were collected via absorbance optics at \(\lambda \approx 236\) nm (interference optics in the case of chemical modification experiments) at 48,000 rpm at 20 °C in an An-60 Ti rotor. Unless specified otherwise, all sedimentation velocity experiments were performed using 0.50 mg/ml Brpt5.5 WT or 5xH85A. Experiments were run overnight, usually around 20 h, at which point all protein was sedimented. Data were analyzed using SEDFIT’s continuous c(s) distribution model (28), SEDANAL’s wide distribution analysis (WDA) (29), or DCDT+ version 2.4.3 (30, 31).

Sedimentation equilibrium experiments were performed using protein dialyzed into the specified ZnCl\(_2\) concentration in 50 mM MOPS, pH 7.2, 50 mM NaCl. After dialysis, protein concentrations were adjusted to \(\approx 0.50, 0.15,\) and 0.05 mg/ml and loaded into a six-channel 1.2-cm centerpiece. Samples were centrifuged at 10,000, 13,000, 17,000, 24,000, and 37,000 rpm for 24 h each, which provided ample time for equilibration of the monomer species to occur at each speed. Raw data were trimmed using WinReedit V0.999 and then fit using WinNonlin V1.080. Data from at least three speeds and three loading concentrations were used for analysis for each Zn\(^{2+}\) concentration. Partial specific volumes were estimated using SEDNTERP (32). Buffer density and viscosity was unavailable for MOPS, so default values were used in SEDFIT, DCDT+, and SEDANAL. As a result, distributions are plotted against the apparent
sedimentation coefficient \( (s^\ast) \) instead of \( s_{20,w} \) values adjusted for temperature, density, and viscosity. Weight-average sedimentation coefficients \( \langle s \rangle \) were calculated from 0 to 15 \( s^\ast \) in SEDFIT. For DCDT+, \( dc/dt \) was computed using the “auto adjust” function to select which scans should be used. The \( \langle s \rangle \) values were calculated from integration over the entire distribution of \( s^\ast \) for each data set. For SEDANAL’s WDA, the range of integration for calculating \( \langle s \rangle \) was from 1 to 20 \( s^\ast \).

**Analysis of linked equilibria**

Experiments were designed and analyzed based on analysis of linked equilibria for Brp1.5 and Brp2.5, discussed elsewhere (9, 19). Data sets shown in Fig. 3 were collected at ZnCl2 concentrations of 1.50, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50, 3.75, 4.00, 4.25, 4.50, 5.00, 5.50, and 6.00 mM, but 1.50 and 6.00 mM ZnCl2 were excluded due to the lack of sufficient dimer or tetramer species, which prevented accurate measurements of both log \( K_{12} \) and log \( K_{14} \) within WinNonlin. In other words, only data sets that produced both log \( K_{12} \) and log \( K_{14} \) measurements were used.

To determine the number of ligand molecules bound or released upon a ligand-dependent equilibrium event, the following equation was used (33).

\[
\Delta Y = \frac{\delta \log K}{\delta \log [Y]} \quad \text{(Eq. 1)}
\]

Here, the number of ligand molecules (Zn\(^{2+}\) ions in this case) bound or released is represented by \( \Delta Y \). This depends on the association constant, \( K \), for a given equilibrium event occurring in the presence of a given ligand concentration, \( [Y] \), in molar units. Thus, this equation was applied to either the dimerization event (using log \( K_{12} \) from WinNonlin) or the tetramerization event (using log \( K_{14} \) from WinNonlin). In both cases, the slope of a log \( K \) versus log[Zn\(^{2+}\)] plot was determined by a linear regression weighted by 1/variance, yielding the number of Zn\(^{2+}\) ions bound during the assembly event.

To convert the log \( K_{14} \) values to log \( K_{24} \) values, the following equation was used,

\[
\log K_{24} = \log K_{14} - 2 \log K_{12} \quad \text{(Eq. 2)}
\]

where \( K_{14} \) is the overall association constant for formation of the tetramer from the monomer, \( K_{12} \) is the (stepwise) association constant for the formation of the dimer from the monomer, and \( K_{24} \) is the (stepwise) association constant for the formation of the tetramer from the dimer.

Error propagation for log \( K_{24} \) was calculated according to Equation 3,

\[
\delta Q = \sqrt{(2 \times \delta a)^2 + (\delta b)^2} \quad \text{(Eq. 3)}
\]

where \( \delta Q \) is the S.D. in log \( K_{24} \), and \( \delta a \) and \( \delta b \) are the S.D. values in log \( K_{12} \) and log \( K_{14} \), respectively.

The S.D. values for log \( K_{24} \) and log \( K_{14} \) were determined from the WinNonlin 95% confidence intervals using Equation 4,

\[
95\% CI = \bar{x} \pm \frac{t \times \sigma}{\sqrt{n}} \quad \text{(Eq. 4)}
\]

where \( \bar{x} \) is the sample mean, \( \sigma \) is the sample S.D., and \( n \) is the sample size (831). The critical \( t \) value, \( t \), was calculated at the WolframAlpha website. The input was “StudentTDistribution[831]”, which returned a value of 1.96282 for the 97.5th percentile for a one-tail \( t \) test. This is equivalent to a 95th percentile for a two-tail \( t \) test. After propagating error for log \( K_{24} \), the 95% confidence interval could be determined using Equation 4. Because 95% confidence intervals for log \( K_{12} \) and log \( K_{14} \) determined in WinNonlin are asymmetric, we assumed that two distributions are present and defined an S.D. based on the lower confidence limit and another S.D. based on the upper confidence limit. We used the average S.D. to determine the variance used for weighting. For log \( K_{24} \), we propagated errors based on the S.D. values from the lower limit and upper limits from log \( K_{12} \) and log \( K_{14} \) to obtain two separate 95% confidence intervals. We then averaged the lower limits from the two confidence intervals to obtain an average lower confidence limit and then used the same process to estimate the upper confidence limit. The linear regression performed on the log \( K_{24} \) plot was also weighted based on 1/variance.

**Chemical modification**

Arginine chemical modification was performed using \( p \)-hydroxyphenylglyoxylic acid (HPG) (G-Biosciences). HPG was dissolved in water at 100 mM. Brp5.5 at 1 mg/ml was dialyzed into 50 mM MOPS, pH 7.2, 50 mM NaCl overnight and then diluted to 0.50 mg/ml in a solution containing 20 mM HPG (final concentration). The reaction was incubated at room temperature for 3 h before loading AUC cells and allowing the samples to equilibrate to 20 °C for 1 h before starting the sedimentation velocity experiment. The Zn\(^{2+}\)-containing samples had 5 mM ZnCl2 added before loading the AUC cells. The unmodified sample was treated identically, but with addition of water instead of HPG.

Tyrosine modification was performed using a final concentration of 2 mM 1-N-acetylimidazole (Sigma–Aldrich). Brp5.5 was prepared in the same way as with arginine modification. 1-N-Acetylimidazole was dissolved in water at 100 mM. The reaction was incubated at room temperature and protected from light for 2 h before adding 5 mM ZnCl2 and performing the sedimentation velocity analysis. For the double modification, both HPG and 1-N-acetylimidazole were added at 20 mM and 2 mM final concentrations, respectively. The reaction was protected from light and incubated at room temperature for 2 h before starting the AUC experiment.

**Circular dichroism**

CD experiments were performed on an Aviv 215 CD spectrophotometer equipped with an Aviv Peltier junction temperature control system and using a 0.5-mm quartz cuvette (Hellma Analytics). For temperature wavelength scans, a single wavelength scan was recorded at 10 °C intervals from 20 to 90 °C and back to 20 °C. Scans were taken from 300 to 190 nm in 1-nm steps, with a 3-s averaging time. The cuvette was not removed in between scans, and a macro was used to perform the

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experiments sequentially. Protein concentrations were 0.50 mg/ml (6.5 μM) and were dialyzed into 50 mM MOPS, pH 7.2, 50 mM NaCl, and then ZnCl₂ was added to a final concentration of 5.00 mM before loading the cuvette. To convert the machine units, θ, to mean residue ellipticity, [θ], Equation 5 was used. Mean residue weight, MRW, was calculated based on amino acid sequence by ExPASy’s ProtParam (34) for Brpt5.5 and Brpt5.5 5xH85A separately, l was 0.05 cm, and concentration, c, was in mg/ml.

\[
[\theta] = \frac{\theta \times MRW}{10 \times I \times c} \quad \text{(Eq. 5)}
\]

For temperature-dependent experiments (Fig. 7), the protein concentration was 1.00 mg/ml, and samples were dialyzed in 50 mM MOPS, pH 7.2, 50 mM NaCl containing 3.50 mM ZnCl₂. A temperature equilibration time of 2 min, averaging time of 3 s, and 0.5 °C interval was used, which resulted in a similar time duration compared with the DLS experiments.

Thermal denaturation experiments (Fig. S7B) were performed with 20 μM Brpt5.5 WT or 5xH85A in 50 mM MOPS, pH 7.2, 50 mM NaCl. The data were converted to mean residue ellipticity and were fitted in SigmaPlot 12.5 (Systat Software, Inc.). The data were fitted to a two-state transition between a folded and unfolded monomer, correcting for pre- and post-transition linear changes as a function of temperature. The fitting assumed no change in the heat capacity between the folded and unfolded states (35).

**Turbidity assay**

A BioMate 3S UV-visible spectrophotometer was used to record the absorbance (or light scattering) at 280, 400, and 700 nm. Protein concentrations were confirmed using the 280-nm absorbance reading before Zn²⁺ additions. A 200-μl sample of 0.50 mg/ml (6.5 μM) Brpt5.5 or Brpt5.5 5xH85A, which had been dialyzed in 50 mM MOPS, pH 7.2, 50 mM NaCl, was added to a quartz microcuvette. 500 mM ZnCl₂ was titrated in 1-μl additions, gently shaking the cuvette between each addition and reading.

**Dynamic light scattering**

To follow temperature-induced aggregation, 200 μl of protein dialyzed into 50 mM MOPS, pH 7.2, 50 mM NaCl, 3.50 mM ZnCl₂ was filtered and added to a low-volume quartz microcuvette. Temperature experiments were performed on a Malvern Zen 3600 Zetasizer Nano, using 2 °C intervals with 120 s of equilibration time and three measurements at each temperature with an automatic measurement duration. The temperature-dependent changes in viscosity were accounted for within the Zetasizer software.

**Data availability**

All data described are presented either within the article or in the supporting information.

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Conflict of interest—A. B. H. serves as a Scientific Advisory Board member for Hoth Therapeutics, Inc., holds equity in Hoth Therapeutics and Chelexa BioSciences, LLC, and was a co-inventor on three patents broadly related to the subject matter of this work.

Abbreviations—The abbreviations used are: Aap, accumulation-associated protein; AUC, analytical ultracentrifugation; DLS, dynamic light scattering; RH, hydrodynamic radius; WDA, wide distribution analysis; HPG, p-hydroxyphenylglycolylxol.

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