An Amyloid Organelle, Solid-state NMR Evidence for Cross-β Assembly of Gas Vesicles*§

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Functional amyloids have been identified in a wide range of organisms, taking on a variety of biological roles and being controlled by remarkable mechanisms of directed assembly. Here, we report that amyloid fibrils constitute the ribs of the buoyancy organelles of Anabaena flos-aquae. The walls of these gas-filled vesicles are known to comprise a single protein, GvpA, arranged in a low pitch helix. However, the tertiary and quaternary structures have been elusive. Using solid-state NMR correlation spectroscopy we find detailed evidence for an extended cross-β structure. This amyloid assembly helps to account for the strength and amphiphilic properties of the vesicle wall. Buoyancy organelles thus dramatically extend the scope of known functional amyloids.

Background: The gas vesicles of aquatic microorganisms are hollow proteinaceous shells with remarkable physical properties that enable them to function as floatation organelles.

Results: The gas vesicle subunits associate in a cross-β arrangement.

Conclusion: The gas vesicle wall constitutes a functional amyloid.

Significance: This new category of functional amyloid broadens our understanding of the diverse roles of the amyloid fold.
sistent with duplicated resonances found in the initial study (13), and further experiments revealed that the protein backbone adopts a coil-$\beta$-coil conformation, in close agreement with the secondary structure tendency of the protein sequence (14). Here, we show that the $\beta$-strands of GvpA form the contacts predicted by the model. It thus appears that the gas vesicle wall comprises a helically wound amyloid fibril.

**EXPERIMENTAL PROCEDURES**

**Preparation of Gas Vesicles**—Cells of *Anabaena flos-aquae* (strain 1403/13f, Cambridge Collection of Algae and Protozoa, Cambridge, UK) were grown in an atmosphere containing $^{13}$CO$_2$ and $^{15}$N$_2$ to produce uniformly $^{13}$C- and $^{15}$N-labeled cells. Floating cells were collected and lysed to release gas vesicles that were isolated and washed by repeated floatation as described previously (13). Washed vesicles were collapsed by a pressure pulse, pelleted by centrifugation, and packed into 3.2-mm rotors, with ~24 mg of sample/rotor.

**Solid-state NMR Spectroscopy**—Magic-angle spinning NMR experiments were performed using a custom-designed spectrometer (D. J. Ruben, Francis Bitter Magnet Laboratory, Cambridge, MA) operating at 700 MHz $^1$H Larmor frequency and equipped with a triple-resonance $^1$H/$^{13}$C/$^{15}$N probe (Varian-Chemagnetics, Palo Alto, CA) fitted with a 3.2-mm magic-angle spinning stator. Preliminary correlation spectra were recorded using various schemes for $^{13}$C-$^{13}$C recoupling, including proton-assisted recoupling (PAR) (15), proton-driven spin diffusion (16), dipolar-assisted rotational resonance (DARR) (17, 18), and band-selective radio frequency-driven recoupling (19). PAR and DARR spectra, recorded with 10-ms and 250-ms mixing periods, respectively, presented optimal sensitivity in these uniformly $^{13}$C-labeled samples and were selected for fur-
ther analysis. Two-dimensional spectra were acquired with 256 indirect points, with a 60-ms dwell time and 320 transients/point. High power, two-pulse phase-modulation $^1$H decoupling (20) was applied during the evolution and detection periods.

Structural Modeling—Representations of GvpA assembly based on the asymmetric dimer model were generated using CNS version 1.2 by applying the simulated annealing protocol (21). The structure was constrained by the backbone torsion angles obtained from chemical shift analysis, hydrogen bonds between putative $\beta$-strands, and translational symmetry between dimers. Calculations were performed in three stages, using an NOE potential. High temperature annealing was carried out by performing molecular dynamics at 50,000 K for 15 ps in torsion space starting from randomized coordinates. The NOE and dihedral angle restraints were applied with force constants of 150 kcal Å$^{-2}$ and 100 kcal Å$^{-2}$, respectively. This was followed by fast cooling from 50,000 K to 3,000 K, in steps of 250 K, with an evolution time of 15 ps at each temperature. The final slow cooling, from 3,000 K to 0 K, was carried out in steps of 25 K with an evolution time of 15 ps at each temperature. Typically, a set of 60 structures was generated for each calculation, and the average of these structures was calculated and minimized. The results were visualized with VMD (22).

RESULTS

As shown in Fig. 1, the proposed model presents four different interfaces between strands, two intramolecular and two intermolecular. The intramolecular interfaces are between the N- and C-terminal portions of the $\beta$-hairpin within each of the two types of monomers. The intermolecular interfaces are between the N(C)-terminal segment of the hairpin of one monomer and the N(C)-terminal segment of the hairpin of the adjacent monomer. Having previously assigned 80% of the GvpA resonances in uniformly $^{13}$C- and $^{15}$N-labeled gas vesicles of A. flos-aquae (14), we sought to validate our model by checking for the predicted interactions between $\beta$-strands in long range correlation spectra. For this purpose, multiple sets of $^{13}$C-$^{13}$C dipolar correlation spectra were recorded under different experimental conditions. The hypothetical model guided our analysis by focusing on potential interstrand contacts within 6 Å, the approximate limit of the dipolar interactions probed by our experiments. Of particular significance are contacts between $\beta$-strand residues that are far apart in the primary sequence because they provide strong evidence for interstrand interfaces. For example, as shown in Fig. 1, the model has Ile$^{23}$ opposite Ile$^{47}$ across the intramolecular interfaces of Gly-turn monomers and opposite Ile$^{45}$ across the intramolecular interfaces of Val-turn monomers. Similarly, the model has Leu$^{39}$ opposite Ile$^{47}$ across the intermolecular interface between C-terminal segments and Ile$^{23}$ opposite Leu$^{33}$ across the intermolecular interface between N-terminal segments.

As shown in Fig. 2, contacts corresponding to interactions between the above residue pairs are observed as cross-peaks in the $^{13}$C-$^{13}$C correlation spectra. Numerous other predicted correlations are also observed, as summarized in Table 1 and Fig. 3. In particular, backbone-side chain and side chain-side chain contacts are observed between many pairs of residues opposing one another in the proposed $\beta$-strand interfaces. It is important to note that, although many long range correlations are readily attainable, we analyzed spectra with relatively short mixing times to minimize relayed transfer and ensure that we identify only near-neighbor interactions.

These solid-state NMR correlations represent the first residue-level characterization of the supramolecular architecture of the gas vesicle wall. Taken together, they assert topological constraints that are probably impossible to satisfy in self-consistent fashion outside the extended, antiparallel $\beta$-sheet arrangement of our model. Some observed correlations are difficult to explain without turns in adjacent monomers that are shifted by one residue. For example, Ile$^{45}$ shows correlations with both Ile$^{23}$ and Ile$^{28}$, whereas Ile$^{23}$ shows correlations with both Ile$^{47}$ and Ile$^{49}$. Other observed correlations require inter-
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TABLE 1
Correlations observed in $^{13}$C-$^{13}$C spectra that correspond to interresidue interactions along the four $\beta$-strand interfaces in our model of gas vesicle organization

These correlations are based on resolved cross-peaks assigned according to chemical shifts published previously (14), except for those in the segment Val$^{29}$-Leu$^{33}$, which were identified subsequently. *, correlations found in the DARR spectrum using a mixing time of 250 ms; $\dagger$, correlations found in the PAR experiment using a mixing time of 10 ms.

| G$_c$-G$_n$ | G$_n$-V$_n$ | V$_n$-V$_c$ | V$_n$-G$_c$ |
|------------|------------|------------|------------|
| I47CG2-I23CB$^\wedge$ | I23CD1-L33CA$^*$ | S32CA-I36CD1$^\wedge$ | E37CB-S49CB$^*$ |
| I47CD1-I23CB$^*$ | I23CG2-L33CA$^*$ | S32CB-I36CB$^*$ | L38CB-A48CA$^*$ |
| V46CB-V24CG1$^*$ | V24CG1-S32CA$^*$ | V31CG2-E37CB$^\wedge$ | L38CB-A48CB$^*$ |
| I45CB-I25CG2$^*$ | I25CG2-V31CB$^*$ | V29CG1-L39CG2$^*$ | L38CB-A48CA$^*$ |
| I45CD1-I25CG2$^\wedge$ | D26CB-R30CG$^*$ | A27CB-I41CD1$^\wedge$ | L39CA-I47CA$^*$ |
| R44CB-D26CB$^*$ | D26CB-E42CA$^\wedge$ | D26CB-E42CB$^*$ | L39CA-I47CB$^*$ |
| E42CB-W28CB$^*$ | D26CB-E42CB$^\wedge$ | D26CB-E42CB$^\wedge$ | L39CD1-I47CG2$^*$ |
| H41CG1-V29CG1$^*$ | I25CG2-A43CB$^*$ | D26CB-E42CB$^\wedge$ | L39CD2-I47CB$^*$ |
| I41CD1-V29CG1$^*$ | I41CA-I45CA$^*$ | E42CB-I44CD$^*$ | L39CD2-I47CB$^*$ |
| A40CB-R30CD$^*$ | E42CA-V46CA$^*$ | V24CG1-R44CD$^*$ | L39CD2-I47CB$^*$ |
| L38CG-S32CA$^*$ | A40CA-V46CA$^*$ | V24CG1-R44CD$^*$ | L39CD2-I47CB$^*$ |
| E37CA-L33CB$^*$ | E42CA-V46CA$^*$ | V24CG1-R44CD$^*$ | L39CD2-I47CB$^*$ |
| E37CB-L33CB$^*$ | I23CG2-I45CA$^*$ | V24CG1-R44CD$^*$ | L39CD2-I47CB$^*$ |
| E37CB-L33CG$^\wedge$ | I23CD1-I45CA$^*$ | I23CD1-I45CA$^*$ | I23CD1-I45CA$^*$ |

FIGURE 3. Interstrand correlations between residues across the four different interfaces of the $\beta$-sheet assembly. The four interface labels are as defined in the legend to Fig. 1. The numeral in the circle between two residues indicates the number of observed $^{13}$C-$^{13}$C correlations assigned to that pair (see Table 1).

strand contacts. For example, the extended secondary structure precludes intrastrand contacts between Asp$^{26}$ and Arg$^{30}$ and between Leu$^{39}$ and Ile$^{47}$. Thus, our solid-state NMR results provide intermolecular and intramolecular constraints that strongly support the proposed model.

**DISCUSSION**

Core $\beta$-Sheet Structure of Gas Vesicles—The structural constraints described above indicate that the intermolecular association between GvpA subunits along the winding filament that forms the ribs of the gas vesicle wall is primarily mediated by $\beta$-sheet formation on both sides of the $\beta$-hairpin. Thus, the $\beta$-strands in the middle of the amino acid sequence form the core of the GvpA assembly in the direction of the rib, approximately perpendicular to the long vesicle axis. This is at variance with a recently reported model involving docking of prefolded monomers (23). However, that work made use of a single monomer fold, rather than two folds differing in the location of the $\beta$-turn. Furthermore, that fold was optimized in bulk solvent, rather than at an air-water interface, and it was not allowed to adapt to interactions with neighbors during docking. The result was a two-dimensional array with holes within and between ribs that make it resemble a sieve more than a barrier. It is difficult to reconcile such a structure with the physical properties of the gas vesicle wall.

The question remains what roles are played by the GvpA sequences that flank the $\beta$-strands. In our model, these segments are attached to the edges of the amyloid ribbons at locations corresponding to the interfaces between the gas vesicle ribs (see Fig. 4). Such an arrangement is consistent with the transverse ridges that are observed in electron micrographs and atomic force micrographs of gas vesicles (12). Mass spectrometry shows that the helices and coils of gas vesicles are largely protected from proteolysis (24), and solid-state NMR spectra indicate that they are largely rigid (14). We therefore expect the helices and coils to be constrained by yet-to-be determined interactions with other parts of the assembly, allowing them to hold the ribs together.

The major physical characteristics of the gas vesicle wall, including its architecture, structural strength, and interfacial stability, reflect the general features of the core $\beta$-sheet structure within the ribs. The inter-rib spacing is determined by the length of the $\beta$-strand segments and their inclination from the vesicle axis (see Fig. 1). The strand orientation also sets the hydrogen bonds at an angle that provides equal strength along and transverse to the cylinder axis (8). In addition, the sequences in the $\beta$-strands are such that one surface of the $\beta$-sheet is highly hydrophobic and likely responsible for preccluding water condensation inside the vesicles. Therefore, the cross-$\beta$ core structure of the gas vesicle rib allows for formation of a resilient supramolecular assembly that can stabilize an air-water interface.

Some of the above structural features resemble morphological properties generally found in amyloid fibrils. The cross-$\beta$ assembly along the rib of the gas vesicle wall is similar to the ribbon-like structure formed by amyloid fibrils, which often presents a twist that arises from the inherent left-handed twist of $\beta$-sheets formed by L-amino acids (25). Instead of a twist, the ribs of gas vesicles curl around the long vesicle axis to produce the cylindrical shape of the wall. Whereas the twist of amyloid fibrils thus appears to be an incidental feature of generic extended $\beta$-sheet structures, the curl of gas vesicle ribs serves a specific purpose in the formation and elongation of this organelle. Furthermore, just as the pitch of the twist varies among amyloid fibrils, gas vesicles from different organisms are found with different diameters that depend on the sequence of the GvpA (8). Finally, amyloid fibrils often consist of multiple, lat-
bic-hydrophilic interfaces have been found to enhance the self-

involving in rib-rib interactions that are likely to also include the turn portions of the β-strands.

Located at the edges of the amyloid ribbon, they are well situated to be involved in rib-rib interactions that are likely to also include the turn portions of the β-strands.

Figure 4. Model of the amyloid ribbon segment formed by three GvpA molecules, illustrating possibilities for rib-rib interactions. The α-helical and coil portions of GvpA are included without any tertiary constraints. Located at the edges of the amyloid ribbon, they are well situated to be involved in rib-rib interactions that are likely to also include the turn portions of the β-strands.

The gas vesicle ribs differ from well known amyloid fibrils in two notable respects. First, the β-strands of gas vesicles cross the fibril at 54° to the fibril axis, rather than at a canonical ~90°. As noted elsewhere, this tilt contributes to the mechanical strength of the gas vesicles (8), a feature that is presumably irrelevant for pathological amyloids and relatively unimportant for the other functional amyloids. Second, the β-strands in gas vesicles are arranged in antiparallel fashion, rather than the more common parallel (usually, but not always, in-register) configuration (26). This is another feature that is expected to contribute to the strength of the vesicle wall because the straight hydrogen bonds of antiparallel β-sheets are stronger than the bent hydrogen bonds of parallel β-sheets.

Amyloid Formation and Air-Water Interface—Hydrophobic-hydrophilic interfaces have been found to enhance the self-assembly of a variety of amyloid-forming peptides into β-sheets, both parallel and antiparallel. For example, amyloid-β peptide, associated with Alzheimer disease, has an amphipathic character and forms β-sheets at air-water interfaces (27). Similarly, recent studies have revealed the critical role of hydrophobic-water interfaces in driving fibril formation by islet amyloid polypeptide and α-synuclein, peptides associated with type 2 diabetes and Parkinson disease, respectively (28, 29). In gas vesicles, it is to be expected that the air-water interface developed by the hydrophobic interior of the gas vesicle wall has the reciprocal effect of stabilizing the amyloid structure of the ribs and facilitating the incorporation of GvpA subunits during vesicle growth.

Current models of hydrophobin assembly (30, 31) are based on compact subunits oriented by a hydrophobic patch into the air-water interface, where they join together to form intermolecular β-barrels. Whereas these secreted amphipathic proteins respond to air-exposed environments, the air-water interface in gas vesicles is itself developed by the gas vesicle wall. For this more rigorous purpose, the continuous hydrophobic surface of β-sheets that are co-planar with the air-water interface seems better suited than discrete hydrophobic patches on the surface of β-barrel units and resembles more closely the β-sheets formed by disease-related amyloidogenic peptides at hydrophobic-hydrophilic interfaces.

Function and Secondary Structure Tendencies—The functional fold of GvpA in the gas vesicle wall contains structural elements consistent with the secondary structure propensity of the amino acid sequence. The high level of sequence conservation in these structural elements (13) is indicative of a highly evolved protein sequence selected for structural integrity in the context of a functional intermolecular assembly. This is in contrast to natively globular proteins that form amyloid fibrils under denaturing conditions. In such cases, the secondary structure of the amyloid state tends to deviate from the secondary structure propensity of the amino acid sequence and instead agrees more closely with the elements predicted by the aggregation properties of the sequence (32, 33). On the other hand, several current models of the assembly of functional amyloids appear to be consistent with preservation of the secondary structure tendencies of the amino acid sequence. For example, supramolecular models of CsgA, the main constituent of curli fibers, are based on repeating segments with sequences predicted to adopt β-sheet conformations (34). In the amyloid assembly of hydrophobins, which are soluble and some of which have known monomeric structures, the antiparallel β-sheets present in the monomeric fold are likely to be preserved after polymerization and flexible loops at each end of the cylindrical monomer appear to establish intermolecular interactions. One of these two loops, disordered in monomer form, is predicted to adopt a β-sheet conformation according to sequence analysis and evidently induces a β-sheet conformation in its partner (31). Therefore, the correspondence between sequence tendency and secondary structure in the amyloid form found in GvpA may be a general feature of functional amyloids, reflecting the evolution of these systems to reach and maintain their assembled state.

Gas vesicles are only soluble in highly protic solvents, and the monomers form amorphous precipitates when such solvents are dialyzed away. It is not fully understood how misfolding of GvpA is avoided in the cell, but because the expression of multiple gas vesicle genes is required for gas vesicle formation, other proteins in this gene cluster may transport GvpA and assist its insertion into the growing vesicle. Mechanisms to prevent aggregation have been recognized in other functional amyloids. The Ma component of Pmel17 self-assembles very rapidly, at rates that are several orders of magnitude faster than those of disease-related peptides (35). It has been suggested that these kinetics prevent the accumulation of intermediates that may lead to unwanted structures (5, 35). Hydrophobin SC3 contains several highly conserved disulfide bridges that are responsible for its solubility when fully immersed in the aqueous environment, such that self-assembly only occurs when triggered by a hydrophobic interface (36). CsgA proteins require the expression of additional genes for export, anchoring, and in vivo curli formation, but not for self-assembly once outside the cell. In this system, at least two other proteins function as chaperones to prevent unwanted aggregation of CsgA.
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The assembly of GvpA into gas vesicles seems to be controlled in a fashion similar to that of CsgA assembly into curli, with the further requirement of the de novo formation of an air-water interface.

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