Analysis of tryptic digests indicates regions of GvpC that bind to gas vesicles of *Anabaena flos-aquae*

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The gas vesicles of the cyanobacterium *Anabaena flos-aquae* contain two main proteins: GvpA, which forms the ribs of the hollow cylindrical shell, and GvpC, which occurs on the outer surface. Analysis by MALDI-TOF MS shows that after incubating *Anabaena* gas vesicles in trypsin, GvpA was cleaved only at sites near the N-terminus, whereas GvpC was cleaved at most of its potential tryptic sites. Many of the resulting tryptic peptides from GvpC remained attached to the underlying GvpA shell: the pattern of attachment indicated that there are binding sites to GvpA at both ends of the 33-residue repeats (33RRs) in GvpC, although one of the tryptic peptides within the 33RR did not remain attached. Tryptic peptides near the two ends of the GvpC molecule were also lost. The mean critical collapse pressure of *Anabaena* gas vesicles decreased from 0.63 MPa to 0.20 MPa when GvpC was removed with urea or fully digested with trypsin; partial digestion resulted in partial decrease in critical pressure.

INTRODUCTION

Gas vesicles are the hollow structures that provide buoyancy in bacteria and archaea. The wall of the gas vesicle has two principal components, the gas vesicle proteins GvpA and GvpC. Crystallographic studies of Blaurock and his collaborators provided information on the folding of the main constituent, GvpA, of gas vesicles (Blaurock & Wober, 1976; Blaurock & Walsby, 1976) but there is little direct information on the arrangement of the outer protein, GvpC. We describe investigations using trypsin to digest the outer protein component that provide some further information on which parts are bound to the structure.

In cyanobacteria the gas vesicle has the form of a hollow cylinder closed at each end by a hollow conical cap: the cylinder varies in length; the diameter is uniform within species but varies in different species. The wall of the cylinder and caps is 2 nm thick and is formed by ribs of width 4.6 nm, which in halobacterial gas vesicles (Offner *et al.*, 1998) have been shown to represent turns of a shallow spiral. The gas vesicle is rigid (Walsby, 1982) and highly permeable to gases (Walsby, 1969) but impermeable to liquid water, which is excluded by the hydrophobic nature of the inside surface (Worcester, 1975).

The wall of the gas vesicle is formed entirely of protein (Walsby & Buckland, 1969; Jones & Jost, 1970, 1971). The main constituent, GvpA, a small hydrophobic protein that forms the ribs and spans the wall, accounts for about 90 % of the mass. In *Anabaena flos-aquae* and several other cyanobacteria GvpA is a protein of 70 amino acid residues (Tandeau de Marsac *et al.*, 1985; Hayes *et al.*, 1986). X-ray crystallography shows that GvpA is arranged in two layers of β -sheet within the thickness of the wall, with two β -chains in each layer tilted at an angle of about 55° to the rib axis (Blaurock & Walsby, 1976; McMaster *et al.*, 1996). A model indicates that this angle is generated by alternate chains of seven and eight dipeptides, which would set the chains at 53.8° to the rib and place the hydrogen bonds between chains at close to the angle that provides the greatest stability, 54.7° to the cylinder axis (Walsby, 1994).

The gene for the second gas-vesicle protein, GvpC, was first found in *Calothrix* sp., where it encodes a hydrophilic protein of 162 residues. The sequence indicated four repeats of a 33-residue repeat (33RR) motif between an 18-residue N-terminal and a 12-residue C-terminal sequence; the nucleotide sequence indicates that the 99 bp repeats encoding the 33RRs have an underlying 33 bp repeat, though there is little discernible homology between the encoded 11RRs in the protein (Damerval *et al.*, 1987). There have been no crystallographic investigations of GvpC but secondary structure predictions suggest that the whole molecule is in the form of a long α -helical rod (<http://www.predictprotein.org/>; Rost *et al.*, 2004).

Abbreviations: 33RR, 33-residue repeat; M_i , monoisotopic mass; MALDI-TOF, matrix assisted laser desorption ionization-time of flight.

In *A. flos-aquae*, *gvpC* encodes a larger protein of 193 residues, with five 33RRs between an 18-residue N-terminal and a 10-residue C-terminal sequence. The encoded protein was shown to be a structural component of gas vesicles by sequencing tryptic digests of the purified structures (Hayes *et al.*, 1988). GvpC can be removed from isolated gas vesicles by rinsing in concentrated urea without the structures collapsing; this demonstrates that it is located on the outer surface (Walsby & Hayes, 1988). The GvpC rebinds to the surface if the urea is dialysed away, and quantitative measurements indicate a binding ratio of one GvpC to 25 GvpA molecules (Buchholz *et al.*, 1993), or one 33RR to five GvpAs (Kinsman *et al.*, 1995). When GvpC is removed, the gas vesicles become less stable and the ribs more easily separated; the stability recovers completely, however, when GvpC rebinds (Hayes *et al.*, 1992). It has been suggested that GvpC crosses the ribs, binding them together (Buchholz *et al.*, 1993) but there is no independent evidence of this.

It is likely that each of the five 33RRs in the GvpC of *A. flos-aquae* forms an identical interaction with the underlying crystalline array of GvpA molecules. Information on the orientation of the outer protein can be obtained by exposing the gas vesicle surface to compounds that label or digest proteins. Belenky *et al.* (2004) analysed digested gas vesicles by MALDI-TOF mass spectrometry. They showed that trypsin removed the N-terminal tetrapeptide of sequence AVEK- from GvpA and carboxypeptidase cleaved the last five peptide bonds in -AAVPA from the C-terminal end; these sequences must occur at the outer, water-facing surface of the gas vesicle. Their analyses were performed on gas vesicles rinsed with urea to remove the adhering layer of GvpC. We have analysed isolated gas vesicles retaining the surface layer of GvpC; digestion of these entire gas vesicles has yielded information on which parts of the GvpC molecule are bound to GvpA.

In the unicellular cyanobacterium *Microcystis* sp. there are 10 different *gvp* genes in a linear cluster *gvpA*, *C*, *N*, *J*, *X*, *K*, *F/L*, *G*, *V*, *W* (Mlouka *et al.*, 2004; Dunton & Walsby, 2005). In *A. flos-aquae*, nine of the same genes are found in the same order, but one, *gvpX*, is absent (see Kinsman & Hayes, 1997, and GenBank accession no. U17109). Two of the cyanobacterial genes (*gvpJ* and *gvpK*) encode proteins that have sections homologous to parts of GvpA and may be structural components of the gas vesicle. We have looked for products of these genes in gas vesicles by MALDI-TOF MS.

In halobacteria there are 14 *gvp* genes (Horne *et al.*, 1991), including *gvpA*, which also encodes the main structural protein, and a weakly homologous *gvpC*, which also encodes another structural protein (Offner *et al.*, 1996). Eight of the genes are essential for gas vesicle production (Offner *et al.*, 2000), including two (*gvpJ* and *gvpM*) with parts homologous to *gvpA*. Shukla & DasSarma (2004) showed that antibodies raised to products of five other *gvps* (*gvpF*, *G*, *J*, *L* and *M*) bind to isolated gas vesicles.

METHODS

Cultures of cyanobacteria and isolation of gas vesicles.

Cultures of *Anabaena flos-aquae* CCAP 1403/13f were grown at 20 °C in low irradiance, 5–15 µmol m⁻² s⁻¹, in the medium of Booker & Walsby (1979). The procedures of Walsby & Bleything (1988) were followed for the isolation of gas vesicles. Cells were lysed by osmotic shrinkage in sucrose and the isolated gas vesicles rinsed by repeated centrifugal flotation, followed by collection and further rinsing on membrane filters of 50 nm pore size. Throughout these procedures the preparations were suspended in solutions containing 5 mM NaCN, to prevent the growth of bacteria, and 10 mM K₂HPO₄, to maintain the pH at about 8; this prevents loss of the GvpC and the ensuing weakening of the isolated gas vesicles.

Digestion with trypsin and Staphylococcus V8 protease.

Aliquots of 100 µl of buffered trypsin solution were made up to 6 mg ml⁻¹ in Tris/HCl (Sigma), pH 8. For MALDI-TOF analysis, samples of concentrated gas vesicles were pipetted into the buffered trypsin and incubated for between 1 and 24 h at 37 °C. The gas vesicles were then rinsed three times by centrifugal flotation through buffer containing 5 mM NaCN and 10 mM K₂HPO₄, pH 8. In some samples GvpC was removed by rinsing 50 µl of concentrated gas vesicles three times through saturated urea (~10 M); the urea was then removed by dialysis against deionized water. The gas vesicle samples were then further concentrated to 100 µg ml⁻¹ by centrifugal flotation and treated with Ziptip (Millipore) before MALDI-TOF analysis. For pressure nephelometry, 10 µl samples of the gas vesicle digests were diluted in 3.4 ml of the same pH 8 buffer (see below).

Similar digests were made using Staphylococcus V8 protease. Samples of concentrated gas vesicles (30 µl) were diluted in 70 µl pH 7.5 buffer containing 50 mM NaH₂PO₄ and 1 mM Na₂EDTA. V8 protease (50 µg) was added and the sample mixed. The mixture was incubated at room temperature for 1 h or 16 h. Samples were then rinsed three times by centrifugal flotation through 50 mM NaH₂PO₄ and 1 mM Na₂EDTA, pH 7.5, and prepared for MALDI-TOF analysis.

MALDI-TOF MS. We analysed the molar masses of proteins and peptides by MALDI-TOF MS with a PE Biosystems Voyager-DE STR Maldi-Tof mass spectrometer, using a nitrogen laser operating at 337 nm. The peptides were analysed in positive ion reflector mode, for masses below 10 kDa, using α -cyano-4-hydroxycinnamic acid matrix. Each monoisotopic mass (*M_i*) was recorded with an accuracy of 50–100 p.p.m. Above 10 kDa, peptides were analysed in linear mode using sinapinic acid as a matrix. Mean masses were recorded with an accuracy of 0.1 %.

Pressure nephelometry. The critical pressure distribution of gas vesicles was measured by pressure nephelometry (Walsby, 1973). Measurements (in triplicate) were made on isolated gas vesicles suspended in 5 mM NaCN and 10 mM K₂HPO₄ (Walsby & Bleything 1988), pH 8, which is optimum for their stability (Buckland & Walsby, 1971). Measurements were made on isolated gas vesicles treated as follows: untreated gas vesicles; gas vesicles incubated in trypsin (4 mg ml⁻¹) to cleave, but not remove, the outer GvpC; gas vesicles stripped of the outer protein GvpC by rinsing in saturated (~10 M) urea; gas vesicles treated with urea to remove GvpC and then incubated in trypsin to cut the N-terminal AVEK-peptide from GvpA. The effects of different incubation times in trypsin, from 5 min up to 24 h, were investigated.

RESULTS

We identified proteins and peptides present before and after removal of GvpC, and before and after exposure to trypsin

and V8 protease. The numbers given in the text below and in Table 1 are means of two or three measurements.

Gas vesicles stripped of GvpC

In the first analysis of isolated gas vesicles treated with 10 M urea to remove GvpC, we obtained a single prominent peak with $m/z = 7403$, where m/z is $(M_i + H)^+ / \text{charge}$, compared with an expected M_i of 7393 for GvpA, an error of 0.12 % in linear mode. The mean $\pm$ SD of nine spectra observed was 7393 ± 10 .

Gas vesicles stripped of GvpC, trypsin digest

In urea-treated samples digested with trypsin a second peak appeared, of $m/z = 6962$. This was identified as GvpA minus

the N-terminal tetrapeptide, of sequence AVEK, which has an expected value of 6966. This confirms the finding of Belenky *et al.* (2004) that the tetrapeptide was accessible at the outer surface of the gas vesicle; P. K. Hayes also demonstrated that trypsin cleaved off some of the N-terminal tetrapeptide by isolating and sequencing the peptide (unpublished results).

There was one occurrence (out of three runs), in a trypsin-digested sample not treated with urea, of an additional fragment of $m/z = 1378$ (Fig. 1a). This corresponds with the sub-N-terminal tryptic peptide of GvpA, TNSSSLAEVIDR; it suggests that both ends of this sequence are present at the outer surface of the gas vesicle but, unlike the N-terminal AVEK, this tryptic peptide was not released. The identity of this fragment needs confirmation, however, because we did

Table 1. M_i values of tryptic peptides of *A. flos-aquae* GvpC compared with those of peptides obtained by digestion of gas vesicles: (a) peptides remaining attached to intact gas vesicles; (b) peptides released from them

The numbers given (except those in brackets) are means of two or three measurements.

Peptide	Amino acid sequence	Monoisotopic mass $[(m/z)-1]^*$				Notes†
		Total, from sequence	(a) Trypsin, attached	(b) Trypsin, released	Untreated gas vesicles	
T1	MISLMAK	792.4 [824.4]	824.6	824.6	—	1, 2, 5
T2	IR	287.2	—	287.9	—	2
T3	QEHQSIAEK	1068.5	—	—	—	3
T2 + T3	IRQEHQSIAEK	1337.7	—	1337.6	—	
T4	VAELSLETR	1016.6	1016.5	1016.6	—	
T5	EFLSVTTAK	994.5	—	—	—	
T6	R	174.1	—	—	—	2, 4
T7	QEQA EK	731.3	—	(727.8)	—	2, 3, 6
T6 + T7	RQEQA EK	887.5	—	887.3	—	2
T8	QAQELQAFYK	1224.6	1224.6	1224.4	—	3
T9	DLQETSQQFLSETAQAR	1950.9	1951.8	1951.0	—	
T10	IAQA EK	658.4	—	(658.3)	—	2, 5
T11	QAQELLAFHK	1183.6	1183.6	1183.8	—	3
T12	ELQETSQQFLSATAQAR	1906.9	1907.8	1907.8	—	
T13	IAQA EK	658.4	—	(658.3)	—	2, 5
T14	QAQELLAFYQEV R	1593.8	1593.7	1593.7	—	3
T15	ETSQQFLSATAQAR	1536.8	1536.7	1536.6	—	
T16	IAQA EK	658.4	—	(658.3)	—	2, 5
T17	QAQELLAFHK	1183.6	1183.6	1183.8	—	3
T18	ELQETSQQFLSATADAR	1893.9	1893.8	1894.0	—	
T19	TAQAK	517.3	—	—	—	2
T20	EQK	403.2	(403.2)	—	—	2
T21	ESLLK	588.4	—	—	—	2
T22	FR	321.2	—	—	—	2
T23	QDLFVSIFG	1024.5	—	—	—	3, 5
T22 + T23	FRQDLFVSIFG	1327.7	—	1327.6	—	5

*Values in parentheses indicate a weak signal present in only one analysis.

†1, Two M residues may oxidize [oxidized mass]. 2, Small molecules: signals may be obscured by background of matrix. 3, If N-terminal Q is deaminated to E, mass increases by 1. 4, Single R residue, unlikely to be found. 5, Hydrophobic peptide, may not dissolve. 6, Poor mass agreement.

Fig. 1. Mass spectra of *A. flos-aquae* gas vesicles digested with trypsin: (a) attached GvpC tryptic peptides; (b) detached GvpC tryptic peptides. Mass sizes given are monoisotopic mass (M_i), after deisotoping.

not find it in a urea-treated sample and we did not find the fragment of $m/z = 5606.2$, representing GvpA minus the first two tryptic fragments.

Entire gas vesicles, containing GvpC

Further investigations were confined to gas vesicles carrying the outer protein, GvpC, whose presence was confirmed by bands of $\sim 22\,000$ Da in PAGE (Walsby & Hayes, 1988). In a MALDI-TOF MS spectrum over the mass range $1\text{--}4 \times 10^4$, of gas vesicles containing GvpC, the most prominent peak was at $m/z = 21\,961$ (within 0.05% of the expected M_i for GvpC of 21 972). The only other prominent peaks were at 11 042 (the doubly charged version of GvpC) and at 14 823, which corresponded with a dimer of GvpA (expected value 14 786).

Entire gas vesicles containing GvpC, digested with trypsin

It was expected that after treatment with trypsin the GvpC would be cut at all post-R and -K sites available to the enzyme: those tryptic peptides with sites that bind them to GvpA would remain on the gas vesicles while those lacking binding sites would be released into the digest. Following digestion, the mixture was centrifuged and the floating gas vesicles were separated from the supernatant suspension. The floating gas vesicles were rinsed twice more to remove

unbound peptides. Samples of both the rinsed gas vesicles and the first supernatant were analysed by MALDI-TOF MS. Table 1 lists all the possible tryptic peptides (those terminating in K or R) and the three cases of adjacent pairs of tryptic peptides where the N-terminal peptide constituted only one or two amino acids.

Attached GvpC tryptic peptides. Analysis of the rinsed trypsinized gas vesicles indicated a number of products, most of which were clearly identifiable with tryptic peptides of GvpC (Fig. 1a). Those identified were all within 1 Da of the expected M_i . Many of the tryptic peptides retained on the gas vesicles were from the region T4 to T18 on GvpC. Fig. 2 indicates the disposition of the attached tryptic products in relation to the amino acid sequence of the GvpC molecule and the location of the 33RRs.

Of these attached peptides, the strongest signals were given by the three longest (T9, T12 and T18, each of 17 residues) and the next strongest by the next longest (T15 of 14 residues and T14 of 13 residues). Those peptides considered missing from this middle section were among the shortest, including the single R residue (T6) and four hexapeptides (T7, and the identical T10/T13/T16). There was a small signal at $m/z = 825.6$, which corresponds to T1, the N-terminal peptide, in which both M residues are oxidized. There were no consistent traces of the next two peptides (T2, T3). The

MISLMAKIRQEHQSIAEK	Repeat
VAELSLETR <u>REFLSVTTAKR</u> <u>QEA</u> <u>EQAEK</u> <u>QAQELQAF</u>	1
YKDLQETSQQFLSETAQAR <u>IAQA</u> <u>EQAEK</u> <u>QAQELLAF</u>	2
HKELQETSQQFLSATAQAR <u>IAQA</u> <u>EQAEK</u> <u>QAQELLAF</u>	3
YQEVRETSQQFLSATAQAR <u>IAQA</u> <u>EQAEK</u> <u>QAQELLAF</u>	4
HKELQETSQQFLSATADAR <u>TAQA</u> <u>KE</u> <u>EQKESLLKF</u>	5
RQDLFVSIFG	

Fig. 2. Amino acid sequence of *A. flos-aquae* GvpC showing the five 33RRs. The underlined K and R residues indicate positions where trypsin cut the protein. Tryptic peptides that remained attached to the gas vesicle are shown in bold.

last five tryptic peptides at the C-terminal end (T19–T23) were absent in two of three analyses made but in a third analysis there was a trace of the small T20. Most of the missing peptides at the two ends are short, though T3 and T23 are nonapeptides, of the same length as T4, which did remain attached. The footnotes to Table 1 give reasons why specific peptides might have been missed in the analysis owing to insolubility or lack of charge.

We also performed analyses on gas vesicles incubated for just 1 h in trypsin; the results were largely similar but small signals indicated that small proportions of two of the peptide pairs, T2 + T3 and T22 + T23, remained attached to the gas vesicles.

Detached GvpC tryptic peptides. A similar analysis was made on the first subnatant, containing peptides released from the trypsinized gas vesicles (Fig. 1b; Table 1). The list contains many of the same peptides that were attached to the gas vesicles, indicating that for those, the binding was not firm enough to prevent partial removal. The main differences between the subnatant and gas vesicle samples were in the presence of the three peptide pairs. The largest signal was given by the T2 + T3 peptide pair. Strong signals were also given by T6 + T7 and by T22 + T23 at the C-terminus. These three peptide pairs were absent from the fully trypsinized gas vesicles. A weak peak corresponding to T10/13/16 was found in one of two digests; its identity is uncertain because it matches the mass of a trypsin autolysis fragment. There were several small peaks which could not be identified with any of the expected tryptic products (see below). The largest of these, with $m/z=1138.4$ and 1408.4 , were about the same size as the smallest identifiable peak, peptide 8 with $m/z=1224.4$.

Products of other gvp genes

If the products of other *gvp* genes form part of the gas vesicle structure, it should be possible to recover them from isolated gas vesicles. Some of the possible products might be present in very small quantities (one molecule per rib or

per end) and detectability would depend on the sensitivity of the technique used.

We drew up lists of the M_i values of the other seven Gvps (GvpN, J, K, F, G, V, W) of *A. flos-aquae* to see if any of them were present among the products of MALDI-TOF MS analysis of isolated gas vesicles. None of them were found.

We also listed the M_i values of all the possible tryptic peptides, including adjacent pairs of tryptic products, in these nine Gvp proteins. Apart from the various tryptic products of GvpA and GvpC described above, the only other identifiable peak was a peak of $m/z=2163$, which was either an autolysis product of trypsin itself (2163.3) or the fourth tryptic fragment of GvpJ (2163.2). The latter seems unlikely because there was no signal for the entire GvpJ molecule. No other identifiable fragments were found.

Staphylococcus V8 protease digest

For the 16 h digest with V8 protease the MALDI-TOF spectrum for mass 500–2000 contained no substantial peaks. A few peaks were present in the spectrum of the 1 h digest but none of them corresponded in size with the peptides that would be generated by cleavage at the D and E residues in GvpC.

Changes in critical pressure of gas vesicles

The effects on gas vesicle strength of removing and/or cleaving gas vesicle proteins were investigated by measuring the critical pressure distributions. Buckland & Walsby (1971) showed that gas vesicles of *A. flos-aquae* were markedly weakened by pronase, an unspecific protease that may have digested both GvpC and GvpA.

The untreated gas vesicles isolated from *A. flos-aquae* had a mean critical pressure of 0.63 ± 0.002 MPa (Fig. 3). In all of the following treatments (urea, trypsin digestion), the suspension retained its milky appearance before pressure, indicating that the gas vesicles remained with their gas spaces intact. After gas vesicles had been stripped of GvpC with concentrated urea, the mean critical pressure fell to 0.21 ± 0.001 MPa, as observed in previous investigations (Hayes *et al.*, 1992; Buchholz *et al.*, 1993). Isolated gas vesicles were also weakened, though less so, by exposure to trypsin for 1 h, which partially digests GvpC: the mean critical pressure decreased to 0.37 ± 0.001 MPa. When these trypsinized gas vesicles were subsequently treated with concentrated urea, however, the gas vesicles were further weakened, to 0.20 ± 0.001 MPa, similar to the result with the stripped gas vesicles. When the order of these treatments was reversed, i.e. the gas vesicles were first stripped of GvpC and the remaining GvpA shell then exposed to trypsin, the same result was obtained, a critical pressure of 0.20 ± 0.004 MPa. Evidently, the cleaving of the N-terminal sequence AVEK- did not further weaken the structure.

Fig. 3. Critical pressure distributions of isolated gas vesicles: ■, entire gas vesicles; □, exposed to trypsin for 1 h, to cleave but not remove GvpC; ●, stripped of GvpC by 10 M urea; ○, digested with trypsin and then stripped of the tryptic peptides; ▲, stripped of GvpC and then digested with trypsin to cleave the N-terminal peptide from GvpA.

The effects of different exposure times to trypsin are shown in Fig. 4. After complete digestion (24 h) the critical pressure had fallen to 0.20 MPa, the same value obtained after removal of the intact or partial tryptic fragments. The time-course indicated that significant changes had occurred after only 5 min exposure to trypsin (critical pressure 0.47 MPa). Complete digestion required digestion for over 5 h (critical pressure 0.23 MPa after 5.2 h).

DISCUSSION

The results in Table 1 provide information on which parts of the attached GvpC molecule are available for cleavage by trypsin. They also indicate which parts of the cleaved GvpC remain attached to the gas vesicle and which parts are released.

Fig. 4. Mean critical pressures of isolated gas vesicles exposed to trypsin for different times. Error bars indicate 95% confidence limits (most are smaller than the symbols).

Orientation of GvpC: sites available for cleavage

We first consider the evidence on which tryptic sites on GvpC are available for cleavage by the enzyme, with the idea that this might distinguish those facing out from those facing in towards the gas-vesicle surface, which might be inaccessible to the enzyme.

The list of tryptic peptides found indicates that, although not all of the possible products were obtained, nearly all the tryptic sites were cleaved. For example, while peptide T3 was not obtained, the flanking peptides T2 and T4 were, indicating cleavage at both ends of T3; the occurrence of the peptide pair T2 + T3 confirms cleavage at the end of T3. There is some doubt about whether trypsin cleaves after both the adjacent K and R residues at the end of peptides T5 and T6, because T5 was not obtained, T6 is indistinguishable from signals given by the matrix and the evidence for T7 is uncertain (present in only one sample, poor mass agreement); however, the peptide pair T6 + T7 confirms that a cut occurs after the K residue at the end of T5. There is strong evidence for cleavage of all the next tryptic sites up to T18; a candidate peak for T20 in one sample extends this to the end of T20. The strong signal for the peptide pair T22 + T23 indicates that the site at the end of T21 was cut even though T21 was not obtained but it is uncertain if a cut occurs after the R at the end of T22, as the evidence for a separate T23 is poor.

These results suggest that, at the most, only two of the tryptic sites (post-T6 and -T22) are inaccessible to the enzyme. It is not possible that all K and R residues are arranged facing outwards on a continuous α -helix because there are examples of R/K residues at intervals of two, three, five and six residues, which must face in different directions. The results suggest that trypsin is able to reach residues at all points on the open helix as the protein vibrates dynamically on the gas vesicle surface. It is therefore not possible to obtain information on the orientation by trypsin digestion. Speculations on structure are made below, but for evidence, crystallographic studies are needed.

Regions of GvpC that bind or do not bind to GvpA

The second purpose of these experiments was to determine which parts of the GvpC were bound to the underlying GvpA gas vesicle by determining which tryptic peptides remained attached to the gas vesicle and which were rinsed off from it. In summary, the results indicated that there are few tryptic peptides that do not bind to some degree, but of the many that do, none bind as strongly as the entire, undigested GvpC, which remains strongly attached in water. It is difficult to determine the ratios of bound to unbound peptide, or the ratios of different peptides, because several factors – e.g. the peptide's size, charge and solubility – affect the signal strength, but we comment on the major differences. For a few of the peptides no signal was detected,

either because cleavage was incomplete or because the peptide did not fly in the mass spectrometer. The evidence on binding of the different tryptic peptides is considered.

The tryptic peptide T1 was not initially recognized: oxidation of the two M residues increases its mass from 792.4 (no corresponding signal) to 824.6. Small signals with this mass were found in samples of both the floating gas vesicles and the subnatant rinsing.

The peptide pair T2 + T3 is evidently from a region that does not bind because it occurs exclusively in the subnatant, where it gave the strongest signal (Fig. 1b). The single T2 peptide occurred only in the subnatant: this dipeptide, once cleaved off, would be too small to remain bound, but the fact that it forms part of a larger peptide pair that does not bind suggests that in the intact protein it does not form part of a binding site.

The first of the five 33RRs shows lower homology to the 33RR consensus sequence than the rest but nevertheless resembles them in the pattern of binding. The first part, T4, occurred in both the bound and subnatant fractions. There was no evidence for T5, which was missing in both fractions. The exclusive presence of the peptide pair T6 + T7 in the subnatant suggests that this region of GvpC does not bind.

There are three conserved classes of tryptic peptides in the 33RRs, of which two remain attached to the gas vesicle. The first class comprises the four products T8, T11, T14 and T17 containing the sequence QAQELXAF (Fig. 2), which all give strong signals in the gas vesicle fraction and somewhat smaller signals in the subnatant. The second class comprises the adjacent four products T9, T12, T15 and T18 containing the sequence ETSQQFLSXTAXAR (Fig. 2), which give the strongest signals in the gas vesicle fraction and smaller signals in the subnatant. Together, these two classes constitute a continuous run of 27 of the 33 residues that binds to the gas vesicle, perhaps at these conserved sequences.

It was previously suggested that each of the five 33RRs in the *Anabaena* GvpC molecule would interact in the same way with an underlying repeating structure on GvpA at the gas vesicle surface: binding might occur either at the 33RRs or at the peripheral N- and C-terminal sequences, with the 33RRs acting as spacers (Buchholz *et al.*, 1993; Kinsman *et al.*, 1995). This investigation shows that regions within the 33RRs bind to GvpA and therefore the 33RRs are not simply unbound spacers.

Each of the 33RRs contains a third class of tryptic peptide, which includes the T6 + T7 peptide pair containing the sequence QAEK, the three identical peptides T10, T13, T16 of sequence IAQAEK, and T19 containing AQA (Fig. 2). No trace of any of these was found in the gas vesicle fraction but some of them appeared in the subnatant: T6 + T7 was in all samples; a weak peak of mass 658.3, which corresponds to either T10/13/16 or a trypsin autolysis fragment, occurred in some samples; T19 was absent from all of them. It is

concluded that these peptides are from regions that do not bind as strongly to the gas vesicle.

There is little evidence of bound or released peptides from the region covered by peptides T19 to T21 but the peptide pair T22 + T23 from the C-terminal end of GvpC provides the strongest signal in the subnatant and indicates that this region of GvpC is not strongly bound to the gas vesicle. Kinsman *et al.* (1995) found that recombinant *Anabaena* GvpC lacking the last 10 residues did not bind to stripped gas vesicles whereas the protein containing the sequence did bind. Perhaps the C-terminal region is required to locate the protein correctly but once attached does not play much part in its binding.

Speculations on binding of GvpC to GvpA

Three types of interactions might link the two proteins – ion pairs, hydrophobic interactions and hydrogen bonds. Ion pairs form between positively charged residues (H, K, R) and negatively charged ones (D, E); they may be of minor importance in binding GvpC because salt solutions, which compete for these charges, affect gas vesicle stability only at high concentrations (Buckland & Walsby, 1971). Hydrophobic interactions occur predominantly between the less water-soluble residues: A, V, L, I and F in GvpC (P, C and W are absent from GvpC and M is absent from the 33RRs). Hydrogen bonds require a donor (K, R, H) and an acceptor (D, E); Q, N, S and T can act as both simultaneously. Q is the best hydrogen-bonding candidate, providing the amide on a longer side chain. (The listing of the tryptic peptides in Table 1 draws attention to a previously unnoticed feature of the GvpC amino acid sequence: with the sole exception of peptide T15, in every tryptic peptide within the 33RRs, the third residue after each K/R is a Q.) Concentrated urea, which removes GvpC from the gas vesicle (Hayes *et al.*, 1992), can weaken all three types of interaction: the two amino groups of urea form ion pairs with carboxyls on acid residues; urea weakens hydrophobic interactions by chaotropic interference; and urea provides competing sites for hydrogen bonds.

Whatever the type of interaction, those residues that bind GvpC to the gas vesicle must be located adjacent to GvpA. If the chain of 33RRs is in the form of a continuous α -helical rod (which requires confirmation), the equivalent interactions in each successive 33RR must occur on the same side of the helix. The angular distribution of residues along the α -helical 33RRs of *Anabaena* GvpC is analysed using a polar plot of the 33 residues in the consensus sequence (Fig. 5), arranged at an angle between residues of 98.2° (see below). Of those able to form ionic bonds, the positively charged K, K and R residues are distributed in different sectors but the four negatively charged E residues are all on one side of the helix (the upper half in Fig. 5). Calculations based on amino acid composition indicate that GvpA has an acidic isoelectric point and is negatively charged at cellular pH values. The hydrophobicity of the interior surface of GvpA suggests that the residual negative charge is located at the

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