Antibodies to the N-terminal sequence of GVPa bind to the ends of gas vesicles

R. S. POWELL, A. E. WALSBY,* P. K. HAYES and R. PORTER

Department of Botany, University of Bristol, Woodland Road, Bristol BS8 1UG, UK

(Received 26 March 1991; revised 20 June 1991; accepted 25 June 1991)

Antibodies were raised against intact gas vesicles of *Anabaena flos-aquae*, and against a synthetic peptide (GVPaNT) whose sequence is identical to the N-terminal region of the main gas vesicle protein, GVPa. A two-stage centrifugation procedure is described for separating gold-labelled antibodies bound to gas vesicles from unbound antibodies. The GVPaNT antibody bound to gas vesicles that had been previously rinsed with SDS to remove the outer gas vesicle protein, GVPc. Treatment with this antibody caused the gas vesicles to aggregate together end-to-end rather than side-by-side. The binding of the anti-GVPaNT-immunogold particles to the gas vesicle was restricted to the conical ends of the structure. These observations indicate that the sequence to which the GVPaNT antibodies were raised, residues 1 to 13 of the GVPa molecule, is exposed only at the outer surface of the cones and that it is normally obscured by GVPc. As GVPa forms both the conical ends and the cylindrical mid-section of the gas vesicle, exposure of the N-terminal sequence only in the cones must be due to differences in the contact between adjacent GVPa molecules in the central cylinders and end-cones.

Introduction

Two distinct types of protein have been found in the gas vesicles of cyanobacteria (Walsby & Hayes 1988; Hayes *et al.*, 1988). The principal constituent, GVPa, is a protein of 70 amino acid residues; it is largely hydrophobic, but it has hydrophilic sequences at each end (Tandeau de Marsac *et al.*, 1985; Hayes *et al.*, 1986). The second constituent, GVPc, is a larger hydrophilic protein containing a number of highly conserved repeats of 33 amino acid residues (Damerval *et al.*, 1987; Hayes *et al.*, 1988). The proportions of the two proteins in the gas vesicles isolated from *Anabaena flos-aquae* have been determined from quantitative analyses of amounts of three of the five amino acids that occur exclusively in one protein or the other: by mass, GVPa forms about 92%, and GVPc, 8% of the total protein present (Hayes *et al.*, 1988; Walsby & Hayes, 1988).

The hollow cylindrical structure of the cyanobacterial gas vesicle is formed from ribs 4–6 nm wide that represent either stacks of hoops or the turns of a shallow spiral (see Walsby, 1989). GVPa forms these ribs; the volume of the unit cell that repeats along the ribs, 9.46 nm³ (Hayes *et al.*, 1986), corresponds with the size of the GVPa molecule, 7.4 kDa. The essential ribbed structure remains intact after the gas vesicles have been rinsed with a solution of 1% SDS, which leaves GVPa

but removes GVPc (Walsby & Hayes, 1988). It has been concluded from this that GVPc must be on the outside of the structure.

In the archaeote halobacteria (Woese *et al.*, 1990) there are gas vesicles of homologous form (Larsen *et al.*, 1967; Stoeckenius & Kunau, 1968; Simon, 1981), which contain a number of homologues of GVPa (DasSarma *et al.*, 1987; Horne *et al.*, 1988; Surek *et al.*, 1988) and may contain a protein analogous to GVPc. These organisms also have several other gene products that appear to be involved in gas vesicle production (Jones *et al.*, 1989), though it is not yet known whether they form structural components of the gas vesicles.

We describe here a method for investigating the presence and location of different protein epitopes in gas vesicles by immunocytochemistry with gold-labelled antibodies. A feature of our method is the quantitative separation of labelled gas vesicles from unbound antibodies and gold particles by centrifugally accelerated flotation. We have investigated the binding to gas vesicles of antibodies raised against intact gas vesicles and of an antibody, anti-GVPaNT, raised against a synthetic peptide with the sequence AVEKTNSSSS-LAE, the first 13 residues at the N-terminus of GVPa (Hayes *et al.*, 1986). The antibody binding was investigated using both entire gas vesicles and gas vesicles from which GVPc had been removed, in order to expose

potential binding sites obscured by the outer protein. We report here a highly specific binding pattern for the anti-GVPaNT antibody, which indicates that this technique, if extended to other parts of the sequence, may give detailed information on the folding patterns of the proteins within the structure.

Methods

Isolation of gas vesicles. Intact gas vesicles were isolated from the cyanobacterium *Anabaena flos-aquae* CCAP 1403/13f using the techniques described by Walsby (1974) and Hayes (1988), with the modification that 5 mM-NaCN was added to the lysate and all rinsing solutions to prevent microbial contamination. Samples of gas vesicles were treated to remove GVPc by mixing 20 µl aliquots with 2 µl 10% (w/v) SDS in 0.5 ml capacity Eppendorf tubes and recovering the gas vesicles that remained intact by centrifugally accelerated flotation, as described by Walsby & Hayes (1988). The treated gas vesicles were then rinsed twice with 40 µl water, being collected again by centrifugation after each rinsing. Any remaining traces of free SDS were removed by passage through a 1 ml affinity column of Extractigel-D (Pierce Chemical Company). The concentration of gas vesicles in suspensions was determined from measurements of their pressure-sensitive optical density (PSOD); at a wavelength of 500 nm a PSOD of 1 cm⁻¹ indicates a gas vesicle protein concentration of 47.9 µg ml⁻¹ (Walsby & Armstrong, 1979).

Antibodies. The peptide representing the N-terminal sequence of GVPa (see Introduction) was synthesized by P. J. Barker (at the AFRC Institute of Animal Physiology and Genetics Research, Babraham, UK). It was linked via a C-terminal cysteine to PPD (a purified protein derivative of tuberculin) to increase its antigenicity, and injected into rabbits from which the immune serum was subsequently collected after three booster inoculations. Rabbit antibodies raised against entire, purified gas vesicles (anti-GV antibodies) were those used previously in agglutination studies (Walker *et al.*, 1984). Control experiments were performed with rabbit antibodies raised against protochlorophyllide reductase (Griffiths *et al.*, 1985).

Immunogold labelling of gas vesicles. Samples of gas vesicles, containing 20 µg GVP in a volume of 1.5 µl, were mixed with 3.5 µl of the serum and 5 µl of PBG [phosphate-buffered saline (Oxoid) containing 0.5% bovine serum albumin (Sigma) and 0.1% gelatin USP, 60 bloom (Fluka)], and incubated for 1 h at room temperature with gentle shaking. The mixture was then diluted with an equal volume (10 µl) of PBG and the excess antibody was removed by centrifuging the mixture, at 1500 m s⁻² (153 g) for 20 min, and drawing off 15 µl of the clear suspension from under the floating gas vesicles.

The antibody-labelled gas vesicles in about 5 µl of PBG were shaken gently for 1 h at 20 °C with 10 µl of a suspension of 10 nm diameter colloidal gold particles linked to protein A (Auoprobe EM, Janssen Biotech) diluted 10-fold in PBG. The suspension, which formed a 5 mm deep layer in a 0.5 ml capacity Eppendorf tube, was then centrifuged in two stages. A first stage at 1500 m s⁻² (153 g) for 200 min, followed by a second stage at 13800 m s⁻² (1410 g) for 1 h is recommended. In the first centrifugation stage the gas vesicles (which float up at 10 nm s⁻¹ per g and would be able to rise 18 mm) accumulated in the surface film. None of the gas vesicles would have been collapsed by the low pressure generated at the low acceleration used (see Walsby & Buckland, 1969). The gold particles (excess density 18 × 10³ kg m⁻³, radius 5 nm) would, according to the Stokes equation, have sedimented only 2 mm in the first stage, but would have sunk 5 mm to the bottom of the tube in the second, high-speed centrifugation. The gas vesicles would then

have remained intact during the high-speed centrifugation in the absence of any overlying water column to generate hydrostatic pressure. We found in one run that most of the gas vesicles reached the surface in the first low-speed spin after only 20 min, presumably because they formed aggregates (see Fig. 1), which according to Stokes's law would float more rapidly than individual gas vesicles.

Gas vesicles with gold particles attached will still float up if the number of particles does not cause their density to exceed that of water. The average *Anabaena* gas vesicle has a volume of 2.35 × 10⁻²¹ m³ (Walsby & Bleything, 1988) and a density of 119 kg m⁻³ (Walsby & Armstrong, 1979); it therefore has a displacement weight of 2.1 × 10⁻¹⁸ kg in water. It will consequently lift 219 gold particles, each with a volume of 5.24 × 10⁻²⁵ m³ and a weight in water of 9.4 × 10⁻²¹ kg. Under the conditions of the first centrifugation, we calculated that individual gas vesicles with < 151 gold particles attached would have floated up the 5 mm to the surface in 200 min. In practice it was found that most of the gas vesicles recovered each had only five or fewer gold particles attached.

The floating gas vesicles were removed with a microsyringe, concentrated by centrifugal flotation and finally resuspended in fresh PBG. Samples were mixed with an equal volume of 2% (w/v) uranyl acetate adjusted to pH 7 with 25% (v/v) aqueous ammonia, and 2 µl quantities were transferred to carbon-coated Formvar-covered grids for electron microscopy.

For the anti-GV antibodies an essentially similar protocol was used except: (1) in place of the serum we used 50 µg of IgG purified on protein A coupled to cross-linked agarose beads, according to the instructions of the manufacturer (Pierce Chemical Company); (2) the gas vesicle sample contained only 2 µg GVP; (3) 20 µl of the protein A/colloidal gold conjugate suspension was used.

Results

By Western blotting we previously established that the anti-GVPaNT antibody bound specifically to GVPa (Walsby & Hayes, 1989). We have also shown by Western blotting that the anti-GV antibody bound to both GVPa and GVPc (P. K. Hayes, unpublished).

Without the centrifugal rinsing procedures, described above, it was not possible to distinguish between gold particles associated with gas-vesicle-bound antibodies and gold particles remaining in the preparation as a result of inefficient washing. After rinsing by centrifugation, almost all of the gold particles that remained in the various preparations were attached to gas vesicles.

Gold particles became attached to all parts of gas vesicles that had been challenged with anti-GV antibodies, both in untreated samples (Fig. 1a) and samples that had been pretreated with SDS to remove GVPc (Walsby & Hayes, 1988) (Fig. 1b). In contrast, there was no gold labelling of gas vesicles that had been challenged with antibodies to protochlorophyllide reductase (used here to provide a negative control; see Fig. 1g).

With the anti-GVPaNT antibody there was no gold labelling of the untreated gas vesicles (Fig. 1f), but a specific labelling pattern was observed with the gas vesicles that had been treated with SDS. In this sample the majority of gold particles were found near or in

Fig. 1. Immunogold labelling of gas vesicles isolated from *Anabaena flos-aquae* CCAP 1403/13f. (a) Untreated gas vesicles and (b) SDS-treated gas vesicles, labelled with anti-GV antibodies raised against entire gas vesicles, showing gold particles bound to all parts of the surface. (c–e) SDS-treated gas vesicles labelled with anti-GVPaNT antibodies, raised against the peptide with the N-terminal sequence of GVPa, showing gold particles bound to the gas vesicle ends, and the gas vesicles aggregated end-to-end to form rosettes (c and d). (f) Gas vesicles untreated with SDS, mixed with anti-GVPaNT antibodies; they did not bind to gold-labelled antibodies and they aggregated side-by-side. (g) SDS-treated gas vesicles mixed with a 'control' antibody to protochlorophyllide reductase, which does not bind to the gas vesicles. Arrows identify the positions of some of the gold particles; the bar marker represents 200 nm (magnification the same in all panels).

contact with the ends of the gas vesicles (see Fig. 1 c–e): 64% of the attached particles were within 15 nm of gas vesicles ends (Table 1). Many of the gas vesicles in this sample were found in small groups arranged with their

ends clustered together (Fig. 1 c, d); 32% of the ends were engaged in such contacts (Table 1). This observation implies that some of the antibodies linked gas vesicles together by their ends, causing them to aggregate in this

Table 1. *Distribution of gas vesicles and gold particles in electron micrographs*Figures in parenthesis represent percentages of the total gas vesicle ends or gold particles given in row *a* or *b*.

	After incubation with anti-GVPaNT immune serum		After incubation with preimmune serum	
	SDS-treated vesicles	Untreated vesicles	SDS-treated vesicles	Untreated vesicles
(a) Numbers of vesicles analysed	216	138	131	62
(b) Numbers of gold particles observed	476	24	110	40
(c) Numbers of gold particles bound to vesicles	362 (76)	5 (21)	23 (21)	2 (5)
(d) Numbers of gold particles bound to vesicle ends	303 (64)	0 (0)	8 (9)	2 (5)
(e) Numbers of vesicle ends associated with gold particles	195 (45)	0 (0)	8 (3)	1 (1)
(f) Numbers of vesicle ends in end-to-end contact	140 (32)	20 (7)	16 (6)	10 (8)

way. Many of the gold particles were located within the spaces between these end-to-end clusters (Fig. 1*c, d*), probably attached to the antibodies binding there. A substantial proportion (31%) of the gold particles that were near gas vesicles were near solitary ends (Fig. 1*e*; Table 1) or at the peripheral ends of gas vesicles in clusters. These gold particles must have been specifically bound to the ends. While the majority of the labelled free ends had only one particle attached, some had two (Fig. 1*e*) or three. Overall, 303 gold particles were near 195 ends (Table 1), a ratio of 1.6 particles per end. Some of the solitary gas vesicles had gold particles attached at each end (see Fig. 1*e*).

These patterns of association found between the ends of SDS-treated gas vesicles and anti-GVPaNT-immunogold particles were not found when pre-immune serum was used or with the gas vesicles untreated with SDS (see Fig. 1*f*). The gas vesicles in these samples were aggregated side-by-side rather than end-to-end.

From these specific associations we can draw the following three conclusions on the distribution of anti-AVEKTNSSSSLAE binding sites: (1) there are binding sites for the anti-GVPaNT antibody on the end cones of the *Anabaena* gas vesicles; (2) there are two or more binding sites per end cone; and (3) there are binding sites at both ends of a gas vesicle.

Discussion

The anti-GV antibody preparation contains antibodies that recognize both GVPa and GVPc epitopes. It is possible that both types of protein are recognized at the surface of the untreated gas vesicles. The SDS-treated gas vesicles do not contain GVPc (Walsby & Hayes, 1988) and the antibodies that bind to these treated structures must therefore have recognized epitopes of the GVPa. As these were polyclonal antibodies there may have been recognition of many different types of

epitopes, which would explain why all parts of the gas vesicle surface were recognized.

We have previously speculated (Walsby & Hayes, 1989) that the hydrophilic sequence near the amino terminus of GVPa would be located at the outer surface of the gas vesicle, since experiments that show gas vesicles partition from oil into water demonstrate that the outer surface is hydrophilic (Walsby, 1971). This property may be partly explained by the presence of the very hydrophilic GVPc at the outer surface (Walsby & Hayes, 1988), but the gas vesicles still partition into an aqueous phase when GVPc has been removed by rinsing with SDS (A. E. Walsby, unpublished).

The high proportion of binding of anti-AVEKTNSSSSLAE at the conical ends indicates that there is a sufficient portion of this sequence exposed for antibody recognition at the outer surface of the cones, but there is an insufficient portion exposed in the cylindrical mid-section. The ribs of both the cones and cylinders are formed from GVPa (or the nearly identical GVPd, which has an identical N-terminal sequence: see Damerval *et al.*, 1989). The lack of labelling of the cylinders therefore indicates that less (or none) of the N-terminal sequence is exposed at the surface of the cylindrical portion.

For reasons of surface geometry the nature of the contacts between GVPa molecules must differ in the central cylinders and the end-cones and, as has been previously discussed (Walsby, 1978), parts of the surface of the molecule that are obscured in the cylinder will be exposed as a result of the different contacts that occur in the cone. The possible additionally exposed areas may include (a) the ends of molecules exposed at the 'acropetal edge' of the rib (i.e. the edge of the rib towards the cone apex); or (b) the surface exposed at gaps between adjacent GVPa molecules within the same rib (see Fig. 2). The width of such a gap may depend on the proximity of the rib to the cone apex. Calculations show that the gap could be 1 nm wide in the rib immediately

Fig. 2. Diagrammatic representation of (a) longitudinal section and (b) three cross-sections, at different positions through the cylinder and cone of a gas vesicle, showing the possible extra exposed areas to which the antibody raised against the peptide AVEKTNSSSLAE may bind. (a) The ends of the GVPa molecules (heavy lines) exposed at the acropetal edge of each rib in the cone. (b) The surfaces at gaps between GVPa molecules within the same rib: the width of the gap increases progressively with proximity to the cone tip. The width of the molecules has been exaggerated about sixfold. After Fig. 1 of Walsby (1978).

adjacent to the cone apex, decreasing to 0.04 nm wide six ribs in from the apex.

The observation that immunogold labelling occurs only after treatment with SDS suggests that attachment of GVPc (which is removed by SDS) normally obscures the part of the GVPa N-terminal sequence exposed on the cones.

The specific labelling pattern of the antibody raised against the N-terminal sequence of GVPa demonstrates that the immunogold technique can be used to explore the orientation of the constituent proteins in the structure of gas vesicles. If active antibodies can be raised against other parts of the amino acid sequences of GVPa, and of GVPc, it should be possible to obtain further information on their orientation. As well as sequences at the outer surface, it is possible to expose sequences located across the profile of the wall by labelling the edges of collapsed gas vesicles, and sequences on the inner surface by labelling freeze-fractured gas vesicles or fragments of gas vesicles exploded apart after raising the internal gas pressure (see Walsby, 1989). Our antibody labelling techniques could also be used to investigate which of the various protein products of genes in the *gvp* operons of halobacteria (Horne *et al.*, 1991) are structural components of the gas vesicles. In particular, it might be possible to determine whether the slightly different GVPa proteins encoded by the plasmid and chromosomal genes are located exclusively on the spindle-shaped or cylindrical gas vesicles produced by halobacteria (Surek *et al.*, 1988; Horne *et al.*, 1988).

This work was supported by grants from the SERC to P. K. Hayes and A. E. Walsby and from the Nuffield Foundation to P. K. Hayes. We wish to thank Annette Bees for culturing the cyanobacteria and P.

A. Baker, of the Institute of Animal Physiology and Genetics Research, Cambridge Research Station, Babraham, Cambridge, CB2 4AT, UK, for raising the specific antibodies.

References

- DAMERVAL, T., HOUMARD, J., GUGLIELMI, G., CSISZAR, K. & TANDEAU DE MARSAC, N. (1987). A developmentally regulated *gvpABC* operon is involved in the formation of gas vesicles in the cyanobacterium *Calothrix* 7601. *Gene* **54**, 83–97.
- DAMERVAL, T., CASTETS, A.-M., GUGLIELMI, G., HOUMARD, J. & TANDEAU DE MARSAC, N. (1989). Occurrence and distribution of gas vesicle genes among cyanobacteria. *Journal of Bacteriology* **171**, 1445–1452.
- DASARMA, S., DAMERVAL, T., JONES, J. G. & TANDEAU DE MARSAC, N. (1987). A plasmid-encoded gas vesicle protein gene in a halophilic archaeobacterium. *Molecular Microbiology* **1**, 365–370.
- GRIFFITHS, W. T., KAY, S. A. & OLIVER, R. P. (1985). The presence and photoregulation of protochlorophyllide reductase in green tissues. *Plant Molecular Biology* **4**, 13–22.
- HAYES, P. K. (1988). Gas vesicles: chemical and physical properties. *Methods in Enzymology* **167**, 213–222.
- HAYES, P. K., WALSBY, A. E. & WALKER, J. E. (1986). Complete amino acid sequence of cyanobacterial gas-vesicle protein indicates a 70-residue molecule that corresponds in size to the crystallographic unit cell. *Biochemical Journal* **236**, 31–36.
- HAYES, P. K., LAZARUS, C. M., BEES, A., WALKER, J. E. & WALSBY, A. E. (1988). The protein encoded by *gvpC* is a minor component of gas vesicles isolated from the cyanobacteria *Anabaena flos-aquae* and *Microcystis* sp. *Molecular Microbiology* **2**, 545–552.
- HORNE, M., ENGLERT, C. & PFEIFER, F. (1988). Two genes encoding gas vacuole proteins in *Halobacterium halobium*. *Molecular and General Genetics* **213**, 459–464.
- HORNE, M., ENGLERT, C., WIMMER, C. & PFEIFER, F. (1991). A DNA region of 9 kbp contains all genes necessary for gas vesicle synthesis in halophilic archaeobacteria. *Molecular Microbiology* **5**, 1159–1174.
- JONES, J. G., HACKETT, N. R., HALLADAY, J. T., SCOTHORN, D. J., CHIN-FEN YANG, WAI-LAP NG & DASARMA, S. (1989). Analysis of insertion mutants reveals two new genes in the pNCR100 gas vesicle gene cluster of *Halobacterium halobium*. *Nucleic Acids Research* **17**, 7785–7793.

- LARSEN, H., OMANG, S. & STEENSLAND, H. (1967). On the gas vacuoles of the halobacteria. *Archiv für Mikrobiologie* **59**, 197–203.
- SIMON, R. (1981). Morphology and protein composition of gas vesicles from wild-type and gas vacuole deficient strains of *Halobacterium salinarum* strain 5. *Journal of General Microbiology* **125**, 103–111.
- STOECKENIUS, W. & KUNAU, W. H. (1968). Further characterizations of particulate fractions from lysed cell envelopes of *Halobacterium halobium* and its lysis in medium of low salt concentration. *Journal of Cell Biology* **34**, 355–395.
- SUREK, B., PILLAY, B., RDEST, U., BEYREUTHER, K. & GOEBEL, W. (1988). Evidence for two different gas vesicle proteins and genes in *Halobacterium halobium*. *Journal of Bacteriology* **70**, 1746–1751.
- TANDEAU DE MARSAC, N., MAZEL, D., BRYANT, D. A. & HOUMARD, J. (1985). Molecular cloning and nucleotide sequence of a developmentally regulated gene from the cyanobacterium *Calothrix* PCC 7601: a gas vesicle protein gene. *Nucleic Acids Research* **13**, 7223–7236.
- WALKER, J. E., HAYES, P. K. & WALSBY, A. E. (1984). Homology of gas vesicle proteins in cyanobacteria and halobacteria. *Journal of General Microbiology* **130**, 2709–2715.
- WALSBY, A. E. (1971). The pressure relationships of gas vacuoles. *Proceedings of the Royal Society* **B178**, 301–326.
- WALSBY, A. E. (1974). The isolation of gas vesicles from blue-green algae. *Methods in Enzymology* **31A**, 678–686.
- WALSBY, A. E. (1978). The gas vesicles of aquatic prokaryotes. *Symposia of the Society for General Microbiology* **28**, 327–358.
- WALSBY, A. E. (1989). The gas vesicle: a stable gas-filled structure in bacteria. In *Supersaturation and Bubble Formation in Fluids and Organisms*, pp. 69–103. Edited by A. Brubakk & G. Sundnes. Trondheim, Norway: Tapir Press.
- WALSBY, A. E. & ARMSTRONG, R. E. (1979). Average thickness of the gas vesicle wall. *Journal of Molecular Biology* **129**, 279–285.
- WALSBY, A. E. & BLEYTHING, A. (1988). The dimensions of cyanobacterial gas vesicles in relation to their efficiency in providing buoyancy and withstanding pressure. *Journal of General Microbiology* **134**, 2635–2645.
- WALSBY, A. E. & BUCKLAND, B. (1969). Isolation and purification of intact gas vesicles from a blue-green alga. *Nature, London* **224**, 716–717.
- WALSBY, A. E. & HAYES, P. K. (1988). The minor cyanobacterial gas vesicle protein, GVPc, is attached to the outer surface of the gas vesicle. *Journal of General Microbiology* **134**, 2647–2657.
- WALSBY, A. E. & HAYES, P. K. (1989). Gas vesicle proteins. *Biochemical Journal* **264**, 313–322.
- WOESE, C. R., KANDLER, O. & WHEELIS, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4576–4579.