An Investigation into the Recycling of Gas Vesicle Protein Derived from Collapsed Gas Vesicles

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Gas vesicle protein may account for more than 6% of the cell dry weight and over 10% of the total cell protein in the planktonic cyanobacterium *Anabaena flos-aquae*. The gas vesicles collapsed by rising cell turgor pressure during buoyancy regulation can therefore represent a significant protein pool within this organism. We have looked for evidence of gas vesicle protein recycling. Gas vesicles were isolated from cells of *A. flos-aquae* that had been pressurized and then cultured in a radioactively labelled medium. The new gas vesicles that formed were less highly labelled than the new vesicles formed in cells from a second culture that had not been pressurized. The results suggest that the collapsed gas vesicles are disaggregated and that the constituent gas vesicle protein is reassembled into new gas vesicles. Proof of this will require experiments using *in vitro* assembly systems.

INTRODUCTION

The walls of gas vesicles, which comprise the gas vacuoles of cyanobacteria and other planktonic micro-organisms, are composed of a single type of protein, gas vesicle protein (GVP) Walker & Walsby (1983). The gas vesicles are hollow structures of low density and they provide buoyancy. Some planktonic cyanobacteria, such as *Anabaena flos-aquae*, regulate their buoyancy by controlling the volume occupied by intact gas vesicles. They do this by accumulating new gas vesicles under low light intensity and collapsing existing intact gas vesicles under high light intensity. The collapse of gas vesicles is brought about by a rise in cell turgor pressure generated partly by an increase in the concentration of organic products of photosynthesis (Dinsdale & Walsby, 1972; Grant & Walsby, 1977) and partly by light-stimulated accumulation of potassium ions from the suspending medium (Allison & Walsby, 1981).

GVP is one of the more abundant proteins found in extracts of *A. flos-aquae* (Walsby, 1977), and may account for over 10% of the total protein. When the cyanobacterium is transferred from conditions of low to high light intensity, over 60% of those gas vesicles initially present may be collapsed (Oliver & Walsby, 1984). We have examined the idea that the GVP from these collapsed gas vesicles is recycled. There are several features of gas vesicles that facilitate such an investigation into the turnover of their component molecules. Firstly, it is possible to isolate intact gas vesicles in a highly purified form (Buckland & Walsby, 1971). Secondly, all of the gas vesicles present in a cell may be collapsed by application of pressure; none of the collapsed gas vesicles are subsequently isolated with the intact structures that are recovered by centrifugally accelerated flotation. It is therefore possible to follow the incorporation of label into the structures from a precisely known time. Thirdly, the fact that there is only one type of constituent molecule, GVP, greatly simplifies interpretation of the results.

We report here the results of ¹⁴C-labelling experiments which support the idea that GVP from collapsed gas vesicles is recycled into new intact gas vesicles.

Abbreviations: GVP, gas vesicle protein; PSOD, pressure-sensitive optical density.

METHODS

Organism and culture. The investigation was carried out with the gas-vacuolate strain of *Anabaena flos-aquae* (1304/13f, Culture Centre for Algae and Protozoa, Cambridge, UK) (Booker & Walsby, 1979). It was grown in medium B (without nitrate) described by Armstrong *et al.* (1983).

Conditions for ^{14}C -labelling. A culture, grown for 10 d at 20 °C and under an incident radiant flux of $30\ \mu\text{E m}^{-2}\text{ s}^{-1}$, was diluted to 2.5 l with fresh, sterile medium. Samples were taken for the determination of total inorganic carbon (Golterman *et al.*, 1978), dry weight, total cell carbon and gas vesicle gas space volume. Two 1-l volumes of culture, one of which had been pressurized (13 bar) to collapse any existing gas vesicles, were sealed into 2-l capacity Buchner flasks and flushed with N_2 , so that the oxygen subsequently produced during photosynthesis would not reach toxic levels. The pressure in the overlying gas space in each flask was then reduced to 0.8 bar. Each flask was then supplied with ^{14}C -labelled NaHCO_3 of known specific radioactivity (given in Results and Discussion) and in sufficient quantity ($\approx 100\text{ mg C}$) to allow growth without carbon limitation. These cultures were then incubated at 20 °C and $20\ \mu\text{E m}^{-2}\text{ s}^{-1}$ incident radiant flux for 5 d. At the end of the labelling period, samples were taken for the determination of dry weight, total cell carbon, gas vesicle gas space volume and the amount of radioactivity in the cells. The remaining material was used for gas vesicle isolation.

Dry weight determination. Five replicate 10 ml samples of the culture were pressurized at 13 bar and centrifuged at an acceleration of 14700 m s^{-2} for 15 min. The supernatant was removed and the pellet resuspended in fresh medium and recentrifuged. The resedimented material was suspended in a total volume of 0.5 ml with fresh culture medium, transferred to a pre-weighed aluminium cup, dried at 60 °C for 12 h, and then 100 °C for 24 h before weighing on a Cahn electrobalance. The dry weights were corrected for the salt content of the fresh medium, which was 0.02 mg in 0.5 ml, compared with 0.3 to 1.0 mg of dried organic material.

Determination of radioactivity. Samples (1 ml) of the *A. flos-aquae* culture were mixed with 5 ml of a mixture of methanol and acetic acid (9:1, v/v) and evaporated to 1 ml under a stream of air. To the concentrate was added 10 ml of Scintran Cocktail T (BDH). Up to 40 μl of radioactively labelled purified gas vesicles were mixed directly with 10 ml of the same scintillation cocktail. The radioactivity of the samples was then determined in a Searle Analytical Isocap 300 scintillation counter and corrected for counting efficiency by the channels ratio method.

Determination of gas vesicle gas space volume. The volume of gas vesicle gas space in samples of the cyanobacterial culture was determined directly from the irreversible contraction of the meniscus in the compression tube apparatus described by Walsby (1982). Triplicate measurements were made. The only modification was a water jacket for the compression tube which gave improved thermal stability. From the measurements of Walsby & Armstrong (1979) it is known that a contraction of 1 nl indicates the presence of gas vesicles containing 0.130 $\mu\text{g GVP}$.

Isolation of gas vesicles. The *Anabaena* filaments were concentrated by filtering onto a 3 μm pore size Nucleopore filter. Intact gas vesicles were then isolated from cells lysed in 1.4 M-sucrose followed by centrifugally accelerated flotation, as described by Walsby & Buckland (1969), and detergent washing (Walsby & Armstrong, 1979). The concentration of GVP in the final purified preparation was estimated from readings of pressure-sensitive optical density (PSOD) at a wavelength of 500 nm and a path length of 1 cm: Walsby & Armstrong (1979) have shown that a suspension of intact gas vesicles giving a PSOD of 1.0 contains 47.9 $\mu\text{g GVP ml}^{-1}$; this is equivalent to 25.5 $\mu\text{g GVP carbon ml}^{-1}$ because the carbon content calculated from the known amino acid composition (Walker & Walsby, 1983) is 53.27% of the dry weight of GVP. The radioactivity of the purified gas vesicles was determined as above and from this the specific activity of the carbon in the GVP was calculated.

Rate of gas vesicle reformation. Growth in two 600 ml cultures of *A. flos-aquae*, maintained at 20 °C and 9 to 9.8 $\mu\text{E m}^{-2}\text{ s}^{-1}$ mean irradiance, was monitored over a 3 d period. Samples were removed from each culture for the determination of dry weight and gas vesicle volume at the start of the incubation period and again after 1, 1.25, 2 and 3 d. The gas vesicles present in one of the cultures were collapsed, by application of pressure, immediately after the sample at day 1 had been taken.

Determination of total cell carbon. Samples of *A. flos-aquae* were prepared by centrifugation as described above (see section on dry weight determination). After freeze drying, the carbon content of the pelleted material was measured using a Perkin Elmer 240 CHN analyser.

RESULTS AND DISCUSSION

The overall changes in dry weight, protein and gas vesicle content of *A. flos-aquae* cells that occurred on transfer from culture A, grown on unlabelled bicarbonate, to cultures B and C, grown for 5 d on labelled bicarbonate, are summarized in Table 1. It is seen that there was no significant difference in cell growth in cultures B and C, as estimated from the change in dry weight, but the gas vesicle content of culture C, which had been pressurized to collapse existing intact gas vesicles, had increased more than that in culture B, which had not been pressurized.

Table 1. *Growth and gas vesicle formation in Anabaena flos-aquae*

Values are given for samples collected at the start of the incubation period (*A*), from a sub-culture after 5 d growth in the presence of [^{14}C]bicarbonate (*B*), and from another sub-culture in which existing gas vesicles were collapsed prior to incubation in the presence of [^{14}C]bicarbonate for 5 d (*C*).

Measurement	Quantities (ml culture) $^{-1}$ (95% confidence limits in parentheses)						
	Final culture after 5 d growth			Increase in quantities		Increase relative to initial culture over 5 d (%)	
	Initial culture (<i>A</i>)	Without collapsing vesicles (<i>B</i>)	After collapsing vesicles (<i>C</i>)	Without collapsing vesicles (<i>B</i> - <i>A</i>)	After collapsing vesicles (<i>C</i> - <i>A</i>)	Without collapsing vesicles	After collapsing vesicles
Gas vesicle volume (nl)	16.40 (± 2.78)	40.24 (± 0)	28.69 (± 2.77)	23.84	28.69 (± 2.77)*	145	175
GVP in intact gas vesicles† (μg)	2.14 (± 0.36)	5.25 (± 0)	3.74 (± 0.36)	3.11	3.74 (± 0.36)*	145	175
Cell dry weight (μg)	50.5 (± 5.51)	91.8 (7.96)	89.8 (± 6.02)	41.3	39.3	82	78
Cell protein‡ (μg)	27.4	49.7	48.7	22.3	21.3	82	78
GVP in intact gas vesicles: cell protein (%)	7.7	10.6	7.7	13.9	17.6	—	—

* All gas vesicles were formed after the collapse of existing gas vesicles in the initial culture. The total gas vesicle content is therefore equivalent to the net increase in gas vacuolation during the labelling period.

† Calculated from gas vesicle volume.

‡ Estimated from that proportion of the dry weight accounted for by protein in cells of *A. flos-aquae* grown under conditions similar to those used in the present study (Oliver & Walsby, 1984).

The protein in the gas vesicles accounted for nearly 6% of the total cell dry weight, and over 10% of the total cell protein in culture *B*, and the new GVP in the extra gas vesicles synthesized represented nearly 14% of the new total protein accumulated during 5 d growth. This relative increase in gas vesicle content is probably a response to the decreasing mean light intensity within the growing culture (Van Liere & Walsby, 1982). The GVP in new intact gas vesicles represented an even larger proportion of new protein in culture *C* but as discussed below some of this GVP may not have been newly synthesized.

At the end of the period of growth on ^{14}C -labelled bicarbonate the radioactivity of the cyanobacteria was measured and it was calculated that the specific activity of the extra carbon incorporated in this period did not differ significantly from the specific activity of the carbon supplied in either culture, *B* or *C*. The results of other analyses performed on these cultures are given in Table 2, and from the values obtained it is possible to calculate the proportion of labelled and unlabelled carbon in the gas vesicles present after each treatment.

The specific activity of the carbon in the gas vesicles isolated from culture *B* [$39.58 \text{ Bq } (\mu\text{g C})^{-1}$] was found to be 0.655 times that of the specific activity supplied [$60.38 \text{ Bq } (\mu\text{g C})^{-1}$]. Thus of the 5.25 μg GVP present per ml of culture 65.5%, or 3.44 μg , had formed from labelled carbon fixed after resuspending the *Anabaena* in ^{14}C -labelled medium. This corresponds fairly closely with the net increase in gas vesicles determined by the compression tube measurements, 3.11 μg per ml of culture. (The small difference between these estimates, 0.33 μg GVP per ml, may indicate that a small proportion of the original unlabelled gas vesicles was lost by spontaneous collapse.) Hence, none of the new GVP accumulated in this culture had been synthesized from unlabelled carbon.

In culture *C*, 3.74 μg of gas vesicles were formed per ml of culture after pressurizing and subsequent transfer to ^{14}C -labelled medium. If all of the carbon they contained had come from

Table 2. *Determination of the amounts of labelled and unlabelled GVP that had formed in the presence of ^{14}C -labelled bicarbonate*

(A) Values from samples taken immediately prior to ^{14}C addition. (B) Values obtained from a culture where the gas vesicles were left intact at the time of the ^{14}C addition. (C) Values obtained from a culture where the gas vesicles were collapsed at the time of ^{14}C addition. Where applicable the 95% confidence limits are given in parentheses.

Measurement*	A	B	C
(a) Specific activity of supplied carbon ($\text{Bq } \mu\text{g}^{-1}$)	nil	60.38	56.93
(b) Weight of GVP in intact gas vesicles (μg)	2.14 (± 0.36)	5.25 (± 0)	3.74 (± 0.36)
(c) Weight of GVP-carbon in intact gas vesicles (μg)	1.14 (± 0.19)	2.79 (± 0)	1.99 (± 0.19)
(d) PSOD of original gas vesicle suspension	ND	25.5	23.2
(e) Conc'n of GVP-carbon in isolated gas vesicle suspension ($\mu\text{g } \mu\text{l}^{-1}$)	ND	0.650	0.591
(f) Radioactivity of gas vesicle suspension ($\text{Bq } \mu\text{l}^{-1}$)	ND	25.73 (± 0.73)	21.70 (± 0.72)
(g) Specific activity of carbon in GVP ($\text{Bq } \mu\text{g}^{-1}$)	ND	39.58	36.72
(h) Weight of ^{14}C -labelled GVP (μg)	nil	3.44	2.41
(i) Weight of unlabelled GVP (μg)	2.14	1.81	1.33

ND, Not determined.

* The values given in *b*, *c*, *h* and *i* are quantities per ml of culture. Values were calculated as follows. (*b*) From Table 1. (*c*) $b \times 0.5327 \text{ g C (g GVP)}^{-1}$. (*d*) Calculated from reading of original suspension diluted 166.7 times in a 1 cm pathlength cuvette. (*e*) $d \times 25.5 \mu\text{g GVP-carbon ml}^{-1}$. (*g*) $f \div e$. (*h*) $b \times g \div a$. (*i*) $b - h$.

carbon fixed after transfer it would have had the same specific activity as the carbon supplied, i.e. $56.93 \text{ Bq } (\mu\text{g C})^{-1}$. In fact the specific activity of the GVP carbon was only $36.72 \text{ Bq } (\mu\text{g C})^{-1}$, which is 0.645 of that supplied. This indicates that about 35% (or $1.33 \mu\text{g GVP per ml}$ of culture) of these new gas vesicles had been formed from unlabelled carbon fixed before pressurizing and transfer. The accumulation of unlabelled GVP in the new gas vesicles could be explained in a number of ways.

(1) *Organic carbon recycled from protein turnover.* It is known that in other bacteria many proteins have a limited life-time and are broken down into amino acids by proteases (Pine, 1972). In theory, the unlabelled protein in the new gas vesicles of the pressurized culture could have been synthesized partly from such breakdown products. However, if this had occurred the new gas vesicles in the unpressurized culture should also have contained unlabelled protein. Since they did not this explanation is unlikely to be correct. The same considerations apply to carbon recycled from other reserves, such as glycogen.

(2) *Recycling of GVP via proteolysis and resynthesis.* The amount of unlabelled GVP present in the new gas vesicles formed after pressure treatment ($1.33 \mu\text{g ml}^{-1}$) was equivalent to 62% of the (unlabelled) GVP in the gas vesicles collapsed by pressure ($2.14 \mu\text{g ml}^{-1}$) and hence all of it could have come from the collapsed vesicles. However, if the GVP from collapsed gas vesicles had been first hydrolysed into its constituent amino acids there is no obvious reason why it should have been used to synthesize new GVP in preference to other proteins. The proportion that would enter GVP would have been no more than the proportion that GVP formed of the total protein being synthesized, i.e. only 17.6% of the $2.14 \mu\text{g ml}^{-1}$ (see Table 1), or $0.39 \mu\text{g GVP per ml}$ of culture, substantially less than the $1.33 \mu\text{g}$ of unlabelled GVP observed. These considerations are made with the assumption that there had been a steady rate of cell growth and gas vesicle production.

A higher proportion of unlabelled amino acids entering new gas vesicles could in theory have resulted from an increased rate of GVP synthesis coinciding with a sudden release of amino acids from the unlabelled collapsed gas vesicles. In a separate experiment we followed the time course of gas vesicle production in pressurized and unpressurized samples of a culture. We again demonstrated a higher rate of gas vesicle production after application of pressure but that increased rate was sustained over 2 d and was not restricted to a short period of time (see Fig. 1). The rate of cell growth showed no significant change over the same period in either sample. Again, there is no support for this explanation from these results.

Fig. 1. Growth and gas vesicle accumulation in *Anabaena flos-aquae*. (a) Change in gas vesicle volume, and (b) change in the dry weight of cells per ml of culture. ●, Culture pressurized on day 1 to collapse existing gas vesicles; ○, control incubation (without pressure).

(3) *Recycling of intact GVP molecules.* A third possibility is that GVP from the collapsed gas vesicles becomes reassembled into new intact gas vesicles. This would be entirely consistent with the high proportion of unlabelled GVP found in the new gas vesicles of pressure treated cells and provides the most likely explanation for the observed labelling pattern.

The recycling of GVP indicated by this experiment was not expected. We therefore performed three further experiments of similar design. In each case we observed that unlabelled GVP was incorporated in the new gas vesicles assembled after application of pressure; in the four experiments unlabelled GVP accounted for a mean value of 43.6% of the new gas vesicles formed. In all of the experiments it was also observed that the unlabelled GVP incorporated in new vesicles was equivalent to a substantial percentage (mean value 61.9%) of the GVP in the collapsed gas vesicles and that the amount of gas vesicles assembled in the pressurized culture exceeded that in the unpressurized one (by a mean value of 38.8%) after 5 d. We also performed a further experiment in which the cells were grown initially in the presence of ^{14}C -labelled medium and then resuspended in unlabelled medium. In this experiment ^{14}C -labelled carbon was recycled into the new gas vesicles that formed in similar proportions to the unlabelled carbon recycled in the experiments described above. Together these experiments provide strong evidence for the unexpected recycling of undegraded GVP from collapsed gas vesicles into new intact ones.

If GVP is recycled without degradation it is likely that this would proceed by disaggregation of the protein molecules followed by their reaggregation. Collapsed gas vesicles cannot be reinflated with gas because they are far too permeable to gases (Walsby, 1969) and they are anyway damaged by the separation of their conical end caps (Walsby, 1971). Lehmann & Jost (1971) demonstrated that following collapse of gas vesicles by pressure the new ones that formed showed the same gradual elongation from biconical initials that characterize gas vesicles forming by *de novo* assembly (Waaland & Branton, 1969).

In order to prove the recycling via disaggregation and assembly it might be necessary to perform experiments with *in vitro* systems of gas vesicle assembly supplied with labelled collapsed gas vesicles. Lehmann & Jost (1972) reported a short-lived assembly system from another cyanobacterium, *Microcystis aeruginosa*. Special factors would be necessary because isolated and purified gas vesicles do not reassemble spontaneously after collapse (Walsby, 1971).

Reassembly of GVP from collapsed gas vesicles would be energetically more favourable than proteolysis followed by resynthesis, and there are precedents for such reassembly systems, e.g. microtubules (Snyder & McIntosh, 1976; Hill & Kirschner, 1982) and coated vesicles (Pearse, 1980).

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