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Characterization and function of carbonic anhydrases in the zooxanthellae–giant clam symbiosis

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Carbonic anhydrase (CA) has been purified from the host tissue of *Tridacna gigas*, a clam that lives in symbiosis with the dinoflagellate alga, *Symbiodinium*. At least two isoforms of CA were identified in both gill and mantle tissue. The larger (70 kDa) isoform is a glycoprotein with both N- and O-glycans attached and has highest homology to CAII. It is associated with the membrane fraction while the smaller (32 kDa) is present in the aqueous phase in both tissues. The 32 kDa CA has high homology with mammalian CAI at the N-terminus. Both isoforms cross-reacted with antibodies to CAII from chicken.

Immunohistology demonstrated that the 70 kDa CA is present within the ciliated branchial filaments and cells lining the tertiary water channels in the gills of *T. gigas*. This is consistent with a role in the transport of inorganic carbon (C_i) to the haemolymph and therefore supply of C_i to the zooxanthellae. CA was also detected in mantle epithelial cells where it may also contribute to C_i supply to the zooxanthellae. The hyaline body and nerve tissue in the mantle express the 70 kDa CA where it may be involved in light sensing and nervous transmission.

Keywords: carbonic anhydrase; inorganic carbon; symbiosis; giant clams

1. INTRODUCTION

Tridacnid clams live in symbiotic association with dinoflagellate algae of the genus *Symbiodinium* (= zooxanthellae). The algal symbionts occupy a system of tubes (Mansour 1946*a*,*b*) arising from the stomach of the clam and terminating in a mass of tertiary tubules (Z-tubules) within the siphonal mantle (Norton *et al*. 1992). The mantle tissue encloses numerous haemal sinuses that are filled with haemolymph. This provides the medium for the bidirectional exchange of nutrients between host and symbiont. This includes photosynthetic products, released from the algae, which support the energy demands of their host (Klumpp *et al*. 1992), and inorganic carbon (C_i) which is required for photosynthesis by the dinoflagellate symbionts. C_i is supplied in the form of CO_2 from host respiration but inorganic carbon is also derived from seawater (Yellowlees *et al*. 1993). The haemolymph system of the clam facilitates their delivery to the mantle.

The efficient supply of C_i to the algae is crucial in ensuring a high rate of algal photosynthesis, and therefore the provision of photosynthate to the host. An essential element of this is the rapid equilibrium of CO_2 and HCO_3^-, a reaction that is relatively slow at physiological pH. In biological processes this is accomplished by the enzyme carbonic anhydrase, which increases the rate by several orders of magnitude over the non-catalysed rate. We have already described a role for carbonic anhydrase in the supply of C_i to clam zooxanthellae (Yellowlees *et al*. 1993). The high carbonic anhydrase activity present in haemolymph, gill, and mantle tissue would enable a rapid adjustment of the C_i equilibrium between haemolymph and seawater.

Carbonic anhydrase is essential to the supply of C_i in a number of other carbon-fixing marine symbioses. For instance, Weis and co-workers have proposed a role for the enzyme in the supply of C_i to zooxanthellae in cnidarians (Weis *et al*. 1989) and demonstrated the requirement for carbonic anhydrase in the sea anemone, *Aiptasia pulchella* (Weis 1991). In the cnidarian symbioses, carbonic anhydrase is on the perialgal membrane, the host membrane which surrounds the zooxanthellae (V. Weis, personal communication). Another symbiosis, and one which appears to closely resemble that of giant clams in terms of the role played by carbonic anhydrase, is the hydrothermal vent tubeworm, *Riftia pachyptila*. The plume and trophosome of the tubeworm play similar physiological roles to the gill and mantle in terms of C_i supply to the autotrophic symbionts and the blood/haemolymph is the interface between these organs in both animals (Goffredi *et al*. 1997). Both these organs possess high carbonic anhydrase activity and two isoforms of the enzyme have been identified (Kochevar *et al*. 1993; Kochevar & Childress 1996).

We have now extended our previous study in an attempt to further understand the C_i supply in giant clams (Yellowlees *et al*. 1993). Here we report the partial characterization of three isoforms of carbonic anhydrase from the gill and mantle of the clam, *Tridacna gigas*. Based on immunolocalization and differential levels in symbiotic and aposymbiotic tissue, we suggest a physiological role for

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these partly characterized isoforms in clam gill and mantle.

2. MATERIALS AND METHODS

(a) *Assay of CA activity and specific activity in study specimens*

Tridacna gigas and *Hippopus hippopus* were obtained from the cultured stocks of the Australian Centre for International Agricultural Research Giant Clam Project at the James Cook University Orpheus Island Research Station, Australia. *T. crocea* were obtained under permit from a wild population in Pioneer Bay, Orpheus Island, and *T. maxima* from One Tree Island Research Station, One Tree Island, Queensland, Australia.

Clams were either taken direct from the sea or maintained at the University aquarium until required. Immediately after scission of the adductor muscle, tissues were dissected from the clam and placed on ice for immediate use or stored frozen at −20 °C. Dissected tissues were then homogenized for 30 s in 50 mM veronal-H_2SO_4 buffer, pH 8.3 (1 ml g^{-1} of tissue wet weight). Extracts were centrifuged at 15 000 *g* and the supernatants assayed for both carbonic anhydrase activity and protein.

CA was assayed using the method of Yang *et al.* (1985). The assay solution contained 3 ml of 18 mM veronal-H_2SO_4 buffer (pH 8.4) and was initiated by the addition of CO_2-saturated distilled water (2 ml). The time taken for the pH to decrease from 8.3 to 7.3 was recorded (T) and activity was calculated. Units are defined as $(T_0/T) - 1$ where T_0 is the time taken for the same pH decrease to occur in the absence of enzyme. The effect of the inhibitors *p*-aminomethylbenzene sulphonamide (*p*AMBS) and acetazolamide (AZ) were investigated by including these in the standard CA assay.

Protein was determined by a modification of the method of Bradford (1976) with the protein dye reagent supplied by Bio-Rad Laboratories. Bovine serum albumin (Fraction V) was used as the protein standard.

(b) *Detergent solubilization of CA from the gills of* T. gigas

Gill homogenate was stirred with varying concentrations of Triton X100 in extraction buffer (50 mM veronal-H_2SO_4 buffer, pH 8.3) at 4 °C for 1 h. Homogenates were centrifuged for 10 min at 15 000 *g* in a microfuge. Supernatants were assayed for CA activity.

The cellular location of some proteins can be determined by their solubility in Triton X-114. Proteins that are soluble will fractionate into one of two phases formed when the temperature of a Triton X-114 solution is increased from 4 °C to 37 °C (Sanchez-Ferrer *et al.* 1994). These represent membrane (Triton phase) and cytoplasmic (aqueous phase) proteins. To determine the cellular location of CA using Triton X-114, homogenates of *T. gigas* gill tissues (30 g) in 50 mM veronal-H_2SO_4 buffer, pH 9.0 (150 ml), were used. Triton X-114 (2% v/v) was added and the homogenate stirred on ice for 1 h. This was centrifuged (30 000 *g*, 1 h and 4 °C) and the pellets resuspended in extraction buffer (50 mM veronal-H_2SO_4 buffer, pH 8.3) for CA assay. After centrifugation, proteins containing glycosyl phosphatidyl inositol (GPI) membrane anchors remain in the pellet (Sanchez-Ferrer *et al.* 1994). The supernatant was recovered and phase separation induced following the method outlined by Sanchez-Ferrer *et al.* (1994), by incubation at 37 °C. Phase separation was completed by centrifuging (19 000 *g*, 10 min). Both the supernatant (aqueous phase, containing cytoplasmic proteins) and lower detergent-enriched phase (containing proteins that are anchored to membranes via peptide anchors) were kept for CA assay.

(c) *Purification of carbonic anhydrase*

Gills were dissected from clams and used fresh or stored at −20 °C before use. Gills (40 g) were weighed, cut into small pieces and homogenized in cold 50 mM veronal-H_2SO_4 buffer, pH 9.0 (100 ml). After homogenizing, Triton X-100 (1% v/v) was added and stirred for 3 h at 4 °C before centrifuging the homogenate at 30 000 *g* for 1 h at 4 °C. The supernatant (*ca.* 100 ml) was dialysed at 4 °C for 24 h against two changes of 50 mM veronal-H_2SO_4 buffer (pH 9.0).

The dialysed supernatant was applied at 3 ml min^{-1} to a Whatman DE-52 anion-exchange column (3.5 cm × 15 cm)-equilibrated with 50 mM veronal-H_2SO_4 buffer (pH 9.0), 1% v/v Triton X-100. Proteins were eluted using 1% Triton X-100, 50 mM veronal-H_2SO_4 buffer (pH 9.0), followed by a 0−0.5 M sodium chloride gradient (500 ml) in 50 mM veronal-H_2SO_4 buffer (pH 9.0). Eluent from the column was collected in 10 ml fractions. The elution of proteins and CA from the DE-52 column was followed by monitoring the absorbance at 280 nm and CA activity of the collected fractions.

Pooled ion-exchange fractions were applied to a *p*-aminomethylbenzene sulphonamide (*p*AMBS) agarose affinity column (3 cm × 8.5 cm) at 1.5 ml min^{-1} at 4 °C. Chlorate, a CA inhibitor, and the low pH of the elution buffer (0.1 M sodium acetate, 0.5 M sodium chlorate, pH 5.6) eluted CA from the *p*AMBS-agarose column (Yang *et al.* 1985). Before measurement of CA activity, selected fractions were dialysed against 5 mM veronal-H_2SO_4 buffer (pH 8.3) to remove chlorate ions.

Fractions containing CA were pooled and concentrated to less than 10 ml using an immersible CX ultrafilter (Millipore) with a 10 000 Da exclusion limit. Concentrated CA was dialysed against two changes of 5 mM veronal buffer, pH 8.3 (2 l).

(d) *Electrophoresis*

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970). Molecular mass determinations were made by comparison with molecular mass standards. Gels were stained for proteins using either Coomassie blue R250 or silver stain (Blum *et al.* 1987).

(e) *Phosphoinositol phospholipase-C digestion*

To determine if CA was anchored to the cell membranes via a GPI anchor, a modification of the phosphatidylinositol-specific phospholipase C (PI-PLC) method described by Sly *et al.* (1991) was used to hydrolyse the GPI anchors from membrane-associated enzymes.

Tissue homogenates of gill from *T. gigas* were centrifuged (2000 *g*, 20 min) and the supernatants recovered and centrifuged again (30 000 *g*, 1 h). Pellets from this final centrifugation, containing membrane-associated enzymes, were resuspended in 70 mM triethanolamine-HCl (TEA) buffer (pH 7.5), 0.16% (v/v) Triton X-100 (TEA/Triton buffer) and used in PI-PLC digests.

To ensure that the reaction conditions were suitable for the hydrolysis of GPI anchors a positive-control reaction was carried out in parallel with the analysis. Alkaline phosphatase from rat kidney, which is known to be attached to membranes via a GPI anchor (Ikezawa & Taguchi 1981), was used as the positive control. Fresh rat kidney (three week Sprague–Dawley) was homogenized in TEA/Triton buffer and used directly in

digests. The *Bacillus cereus* PI-PLC (Ikezawa & Taguchi 1981) enzyme was added at $0.025\ \mathrm{U\,ml^{-1}}$ to the gill and kidney TEA/Triton preparations and both were incubated at 37 °C for 12 h. Negative-control reactions, containing no PI-PLC, were run in parallel for each tissue type. Digests were then centrifuged (30 000 *g*, 1 h) and any GPI-anchored enzymes released from the pellets were detected by enzyme assay of the supernatant.

Kidney digests were assayed for alkaline phosphatase activity (10 mM *p*-nitrophenol phosphate, 50 mM Na_2CO_3, pH 10.1) by measuring the production of *p*-nitrophenol ($\lambda_{max}=400$ nm) from *p*-nitrophenol phosphate. Gill digests were assayed for CA activity as previously described.

(f) *Deglycosylation of CA*

Digestion with N-glycosidase F was used to hydrolyse N-linked glycans, while trifluoromethanesulphonic acid (TFMSA) was used to remove O-linked glycan (Edge *et al*. 1981). HF treatment was used for removal of both N- and O-linked glycan (Sairam & Schiller 1979).

(g) *Amino acid sequencing and sequence analysis of CA peptides*

CA peptides for amino acid sequencing were prepared by chemical digestion with cyanogen bromide (CNBr). CA peptides were separated by SDS-PAGE and transferred to ProBlot membrane (Applied Biosystems) in the absence of SDS using the method of Kyhse-Andersen (1984).

Sequencing from blots was done by Dr Dennis Shaw (John Curtin School of Medical Research, Canberra, Australia) on an Applied Biosystems Model 477A Protein Sequencer. Sequencing protocols were standard protocols recommended by the supplier. Additional sequencing was done by Dr Robert Olafson (Department of Microbiology, University of Victoria, Victoria, British Columbia, Canada).

The alignment method used for sequence analysis was the basic local alignment search tool for proteins (BLASTP) (Altschul *et al*. 1990) run against the combined non-redundant GenBank CDS translations, PDB, SwissProt, Spupdate and PIR databases at the National Center for Biotechnology Information (NCBI), USA.

(h) *Preparation of antibodies to carbonic anhydrase from* Tridacna gigas *gill*

Purified CA (1 mg) in Freund's complete adjuvant was intramuscularly injected into a rabbit. Three further intramuscular injections of 1 mg each in Freund's incomplete adjuvant were made at fortnightly intervals. After a further month, a final injection of 100 μg of the 70 kDa CA, purified by preparative-gel SDS-PAGE, was given. Two weeks later, rabbit serum was collected and used for detection of antigen on Western blots and in tissue sections.

(i) *Detection of peptides after Western blotting*

Immunoblotting was performed as described by Harlow & Lane (1988) using the anti 70 kDa CA serum at a dilution of 1:10 000. Antisera to chick CAII was obtained from Dr Linser (Whitney Laboratory, University of Florida, USA) and used at a dilution of 1:2000. For the detection of mannose-containing glycoproteins the method of Clegg (1982), using peroxidase-conjugated concanavalin A, was used.

(j) *Immunohistological analysis of giant clam tissues*

Clam tissues were fixed in marine Bouin's for 24 h before being washed in 70% ethanol. Tissues were dehydrated through increasing concentrations of ethanol, cleared in xylene, and infiltrated with paraffin wax in an automatic tissue processor. Sections (6 mm) were cut from infiltrated tissues, mounted on microscope slides, incubated at 60 °C overnight, and de-waxed in xylene for 5 min. Slide sections were rehydrated through graded ethanol washes and immersed in water. Immunohistological detection of CA using the antiserum against the 70 kDa CA was carried out following the protocols outlined in Ormerod & Imrie (1989).

The primary 70 kDa CA antibody was used at 1:50 dilution, whereas the secondary antibody—swine anti-rabbit immunoglobulins, conjugated to horseradish peroxidase (DakoPatts)—was used at 1:250 dilution. The substrate used for visualization was 3,3′-diaminobenzidine (DAB) which gives a brown product following a positive reaction. Negative-control sections were processed using the same dilution of secondary antibody, without the primary antibody step. A second negative control where the primary antibodies were pre-treated by the addition of an equal volume of *ca*. $1\ \mathrm{mg\,ml^{-1}}$ purified CAs for 1 h, to block the CA-specific antibodies, was also used. Sections were then stained with haematoxylin, mounted under coverslips in DePex (supplied by BDH), and photomicrographed.

3. RESULTS

Significant concentrations of CA were detected in both mantle and gill tissue. However, the concentration of CA in gill extracts was very variable. This was overcome by inclusion of the non-ionic detergent, Triton X-100, in the extraction media. This increased yields 15-fold suggesting that much of the CA may be associated with the membrane (figure 1). The increase in activity cannot be attributed to the activation of CA by Triton X-100 because the addition of 0.5% Triton X-100 to affinity-purified CA from which the detergent had been removed only increased the enzyme activity by 1.5-fold. A similar concentration of Triton X-100 added to tissue homogenate caused a sevenfold increase in activity through solubilization (figure 1).

CA preparations were very stable and no activity was lost from *T. gigas* gill extracts after 25 days storage at −20 °C. However, addition of 15 mM β-mercaptoethanol caused the loss of all activity in the extract when stored under the same conditions.

Both *p*AMBS (0.05 μM to 1 mM) and acetazolamide (0.05 nM to 0.4 μM) inhibited CA activity in *T. gigas* gill and mantle extracts. The acetazolamide I_{50} for both gill and mantle CA fell between 1 and 5 nM; for *p*AMBS it fell between 5 and 10 μM. Inhibition by *p*AMBS suggested the use of an affinity column incorporating this inhibitor as ligand for the purification of CA.

Purification of CA was achieved by first passing a gill extract through a DE-52 column and eluting with an NaCl gradient. CA eluted as a single peak at 250 mM NaCl. Fractions containing CA were applied to a *p*AMBS-agarose affinity column and, following the procedures outlined by Yang *et al*. (1985), were eluted as a single peak (figure 2). This resulted in a 280-fold purification of CA activity (table 1). The purified CA had a specific activity of 3900 units $\mathrm{mg^{-1}}$ of protein compared with that of pure bovine-erythrocyte CA which, assayed under the same conditions, had a specific activity of 8100 units $\mathrm{mg^{-1}}$ of protein.

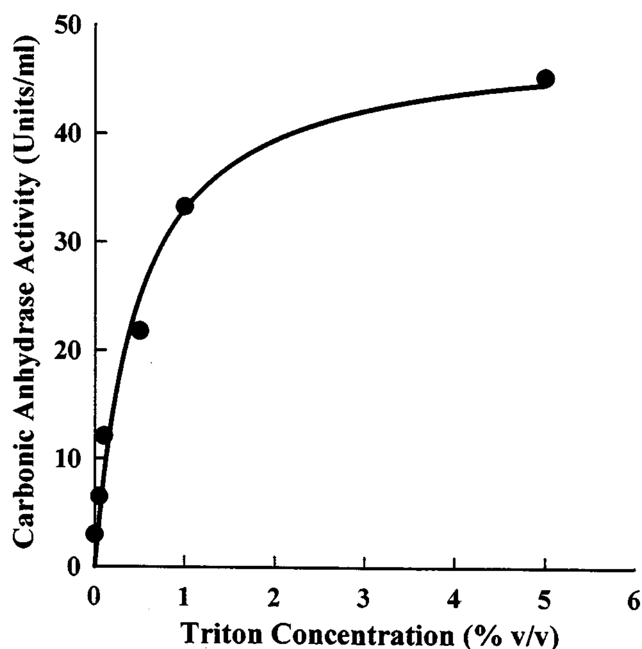

Figure 1. The effect of Triton X-100 on the solubility of carbonic anhydrase from *Tridacna gigas* gill.

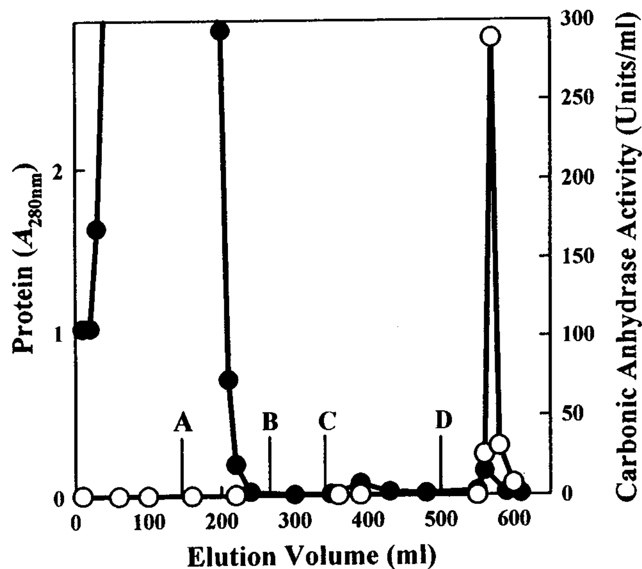

Figure 2. Elution of carbonic anhydrase from *p*AMBS-agarose. (A: 50 mM Bicine buffer, pH 8.5; B: 25 mM Tris-HCl, 22 mM sodium sulphate; C: 25 mM Tris-HCl, 0.3 M sodium chlorate, pH 8.7; D: 0.1 M sodium acetate, 0.5 M sodium chlorate, pH 5.6). Protein (filled circles) and activity (open circles) profiles displayed.

SDS-PAGE analysis of the purified CA obtained from both gill (figure 3) and mantle (not shown) demonstrated the presence of three proteins with molecular masses of 32 kDa, 70 kDa and 200 kDa. The 70 kDa protein was always the major component, with the 200 kDa and 32 kDa species being present in much smaller concentrations. Despite using a range of techniques we were unable to isolate these individual proteins from mantle or gill in a pure, active form.

Later preparations of CA from *T. gigas* gill were purified using affinity chromatography alone, and gave a 179-fold purification with no difference in the final specific activity, but a much higher yield (*ca.* 85%). CAs from the mantle and gill of *T. gigas* could be purified in the absence of Triton X-100 but with significantly lower yields (data not shown).

CA purified from the gill, mantle and digestive mass of *T. gigas*, and from the gills of *T. maxima* and *H. hippopus*, all contained the 70 kDa and 32 kDa peptides, whereas only the tissues from *T. gigas* contained the 200 kDa CA. N-terminal amino acid sequences of the 32 kDa, 70 kDa and 200 kDa CAs purified from giant clams were determined by microsequencing and compared with known CAs (figure 4). Of the large number of CAs which were homologous with the 32 kDa isoform from giant clams the two that were the most similar were mouse CA I (Fraser & Curtis 1986) and *Anabaena* PCC7120 CA (Soltes-Rak *et al.* 1997) (figure 4*a*). The 70 kDa and 200 kDa proteins had identical N-terminal amino acid sequences but showed poor homology to other known CA sequences. Of the proteins that were homologous with the N-terminus of the 70 kDa isoform from *T. gigas*, the only CA detected was CAII from chicken (Yoshihara *et al.* 1987) (figure 4*a*).

Digestion of the 70 kDa CA from *T. gigas* with CNBr produced several peptides, two of which were separated by SDS-PAGE. The amino acid sequences obtained for these two peptides were CNBr1 (PEDWSNLEYYRYLGSLTTP) and CNBr2 (ADISYDSHGNTKQPQ). The N-terminal sequence of the CNBr1-peptide was analysed and found to be homologous to both the chicken CAII (Yoshihara *et al.* 1987) and the CA from *Neisseria gonorrhoeae* (Chirica *et al.* 1997) (figure 4*b*).

Table 1. *Purification of carbonic anhydrase from* Tridacna gigas *gill*

fraction	total protein (mg)	total activity (units)	specific activity (units mg^{-1})	yield (%)	purification
extract	800	11400	14.1	100	1
supernatant	680	7900	11.6	70	0.8
DE-52 cellulose	100	3500	35.3	31	2.5
*p*AMBS-agarose	0.61	2400	3900	21	280

Western blots of clam CA probed with concanavalin A-peroxidase conjugate revealed that the 70 kDa and 200 kDa species were glycoproteins. Deglycosylation of the 70 kDa CA with N-glycosidase F and subsequent analysis of the product by SDS-PAGE (figure 3*b*) and Western blots indicated the deglycosylated CA had a mass of 69 kDa. This protein no longer reacted with the peroxidase-conjugated concanavalin A. The presence of O-glycans was investigated using TFMSA. This caused a reduction in mass (62 kDa), while the digestion of the 70 kDa CA with HF, which hydrolyses both O- and N-linked glycans, reduced the mass to *ca.* 62 kDa.

The possible location of gill CA in the membrane was further investigated by phase separation with Triton X-114. Gill homogenate, containing all three CA isoforms, was treated with 2% v/v Triton X-114, and phase separation was carried out. Of the CA activity solubilized in Triton X-114 at 4 °C, most partitioned into the detergent phase (80%) and the balance into the aqueous phase (20%) after

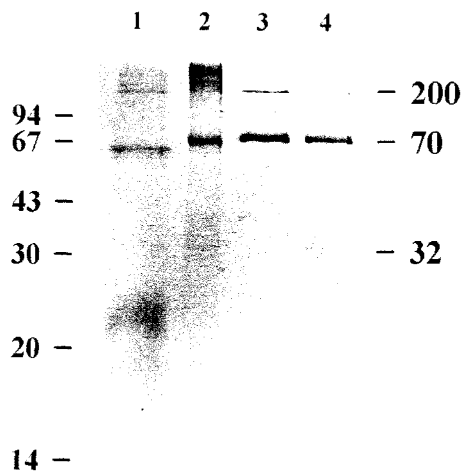

Figure 3. SDS-PAGE analysis of the carbonic anhydrase from *Tridacna gigas*. Lane 1, O- plus N-deglycosylated 70 kDa CA (0.5 μg); lane 2, N-deglycosylated 70 kDa CA (2 μg); lane 3, CA isoforms (1 μg) in Triton X-114 detergent phase (gill); lane 4, purified gill CAs (0.5 μg). Molecular size of standards are indicated on the left and the CA isoforms on the right.

phase separation at 37 °C. SDS-PAGE analysis demonstrated the presence of the 70 kDa and 200 kDa and the absence of the 32 kDa CA in the detergent phase (figure 3*b*).

PI-PLC digestion of crude membrane preparations of both *T. gigas* gill and rat kidney were conducted. PI-PLC treatment resulted in the release of alkaline phosphatase from rat kidney membranes compared with a negative control (without added PI-PLC) indicating reaction conditions were suitable for release of GPI-anchored proteins from cell membranes. No release of CA from gill membranes by PI-PLC digestion could be detected after 12 h. Hence, it seems unlikely that CA is anchored to the cell membranes by PI-PLC-susceptible GPI anchors.

Antibodies raised against the 70 kDa CA from the gill of *T. gigas* reacted with the antigen on Western blots (figure 5*b*). They also recognized the corresponding CA from other clams, including extracts from the gills of *T. maxima*, *H. hippopus*, and *T. crocea* (not shown). However, the antibody did not cross-react with the 32 kDa CA from giant clams (figure 5*a*), nor the 30 kDa CA from bovine erythrocytes.

Antibodies raised in rabbits against the CAII from chicken cross-reacted with both the 70 kDa and 32 kDa CAs from the symbiotic mantle of *T. gigas* and a 32 kDa CA from zooxanthellae. These antibodies also reacted with the CA from bovine erythrocytes (not shown).

Antibodies raised against the CA from *T. gigas* gills were used to detect CA in tissue sections from *T. gigas*. The high molecular mass isoforms were located by the presence of the oxidized DAB product (brown precipitate). Using this method the 70 kDa CA was localized to the apical membranes of the ciliated epithelial cells lining the lamellae and water channels of the gill (figure 6*b*). The enzyme was also found on mantle epithelial cells (figure 6*a*), in nerve tissues (figure 6*c*) and in the hyaline organs of the mantle (figure 6*d*).

Negative-control sections processed without the inclusion of primary antibody remained unreactive to the DAB stain, demonstrating the absence of non-specific reaction by the DAB stain and the secondary antibodies. Sections processed with primary antibody, which were pre-treated with purified CA, remained unreactive to the DAB stain, demonstrating that the staining pattern observed is specific to the CAs in the purified CA preparation.

4. DISCUSSION

The results presented here demonstrate the presence of at least two isoforms of carbonic anhydrase in clam host tissue and provide evidence as to their function in both carbon supply to the symbiosis and light perception.

CA from both gill and mantle was readily purified on a *p*AMBS-agarose affinity column yielding proteins with molecular masses of 32 kDa, 70 kDa and 200 kDa. The most abundant of these was the 70 kDa protein. In the case of the gill tissue we can readily conclude that all three derive from animal tissue and not zooxanthellae as the gills contain insignificant numbers of symbionts. This would suggest that the proteins isolated from mantle are also of animal origin. This is supported by immunolocalization experiments that indicate the 70 kDa and 200 kDa species are not associated with the Z-tubules in the mantle and therefore not expressed by zooxanthellae. The 32 kDa CA would also appear to be an animal protein as we have purified it from the digestive mass of *T. gigas*, a tissue that has few zooxanthellae.

That all are carbonic anhydrase isoforms is indicated by several experiments. First, SDS-PAGE gels show that these were the only three detectable proteins and, since the specific activity of the purified mixture was in the range expected of a pure CA, it is unlikely that another protein could be responsible. Second, in the Triton X-114 preparations the 32 kDa protein was not present yet the CA activity was high, indicating that the 70 kDa and 200 kDa species are indeed carbonic anhydrase. Finally the N-terminal amino acid sequence of the 32 kDa protein has a high homology to other CAs in a range of animals.

The 15-fold increase in CA activity caused by addition of 5% Triton X-100 to tissue homogenates implies that CA is associated with membranes. Consequently, detergent was included in the extraction buffer for CA purification, although at a final concentration of only 1% so as to facilitate the removal of the detergent in subsequent stages of the purification.

Phase separation with Triton X-114 partitioned the 70 kDa and 200 kDa proteins and most of the CA activity into the detergent-rich phase. This is consistent with these CAs being membrane proteins (Sanchez-Ferrer *et al.* 1994). The 32 kDa protein remained in the aqueous fraction of the cell.

The evidence that the 70 kDa CA is membrane-bound is further supported by the immunolocalization of the enzyme to membranes in the gill. Immunohistological procedures demonstrated that this CA is most abundant in the ciliated cuboidal epithelium lining the branchial filaments and tertiary water channels in the gill. The 70 kDa CA does not appear to be attached to the membrane by a PI-PLC-sensitive GPI anchor because PI-PLC digestion failed to solubilize the CA from a gill-membrane preparation. In this respect it is different to CAIV from human lung and kidney where the enzyme is attached to the membrane through a PI-PLC-sensitive GPI anchor (Sly *et al.* 1991).

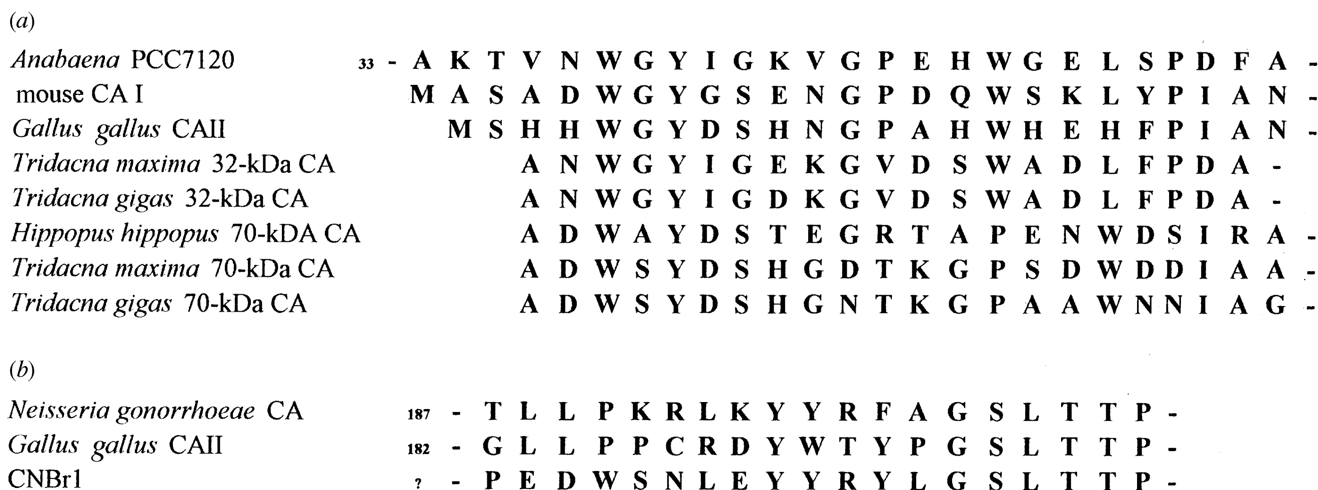

Figure 4. Alignment of the amino acid sequences of carbonic anhydrases from the giant clams with known carbonic anhydrases: (*a*) N-terminal sequences, and (*b*) the CNBr1 peptide from the 70 kDa CA of *Tridacna gigas*.

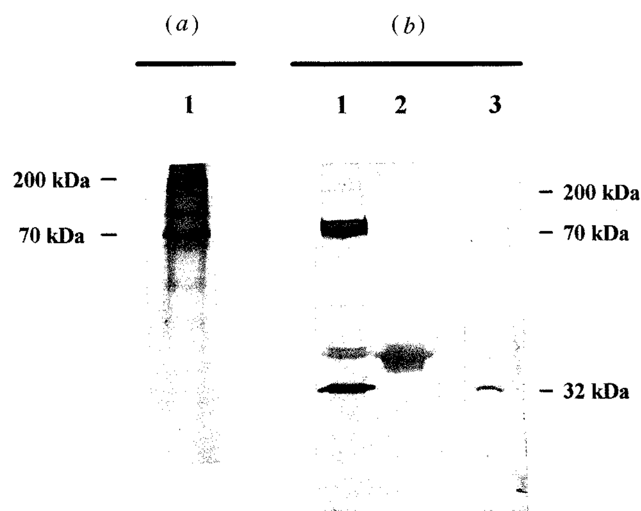

Figure 5. Analysis of carbonic anhydrases from zooxanthellae and the tissues of giant clams by immunodetection on Western blots using rabbit antibodies raised against: (*a*) 70 kDa CA from the gills of *Tridacna gigas*. (lane 1, proteins from *T. gigas* gill (40 μg)); and (*b*) CAII from chicken. (lane 1, proteins from the symbiotic mantle of *Tridacna gigas* (10 μg); lane 2, proteins from the aposymbiotic mantle of *T. gigas* (10 μg); lane 3, crude proteins from zooxanthellae (10 μg) obtained from Dr S. Whitney, James Cook University).

Analysis of the 70 kDa protein indicated that it has both N-linked and O-linked glycan side chains, the N-linked glycans containing mannose residues. Glycosylated CAs are relatively rare, but not unknown. However, CAIV from bovine lung (Carter 1991) has characteristics similar to that of the 70 kDa CA from *T. gigas* gill in that it has a higher-than-usual molecular mass, it is glycosylated and is associated with membranes.

The N-terminal sequence from the 70 kDa CA showed only limited homology with one animal CA (CAII from chicken), whereas the internal amino acid sequence revealed similarities with CAII from chicken and the CA from *Neisseria gonorrhoeae*. However, a BLASTp analysis of the combined N-terminal and an internal amino acid sequence shows that the 70 kDa CA shows greatest homology with CAII from chicken (Altschul *et al.* 1990). This was supported by the cross-reaction of antibodies raised against chicken CAII.

The N-terminal amino acid sequence of the 200 kDa CA was identical to that of the 70 kDa protein, suggesting the possibility that it has the same polypeptide backbone but is conjugated to a larger glycan or lipid component. The possibility of it being a multimer of the 70 kDa protein cannot be discounted.

The antibody raised to the 70 kDa CA cross-reacted with the 200 kDa species but did not cross-react with the 32 kDa CA. However, the antibody raised against CAII from chicken reacted with all *T. gigas* CAs.

The greatest concentration of CA (70 kDa) is on the ciliated cuboidal epithelium lining the branchial filaments and tertiary water channels of the gill. This would catalyse the rapid equilibrium of CO_2/HCO_3^- in the water channels of the gill, thus enabling the movement of CO_2 into the haemolymph when CO_2 concentrations in the haemolymph drop below those of seawater. During the day, algal photosynthetic activity fixes CO_2, depleting haemolymph C_i and increasing the haemolymph pH from 7.3 to $\geqslant$8.1 (Fitt *et al.* 1995). Indeed, during high rates of photosynthesis induced by elevated light levels, the C_i concentration of haemolymph can be reduced to as low as 0.8 mM (D. Yellowlees, unpublished results). This ensures that during the daytime the CO_2 flux is inwards, towards the algal symbionts, as the CO_2 concentration in the haemolymph is below that of seawater with no difference in pH or ionic concentration (and hence pK_a).

The C_i concentration of haemolymph in the dark never exceeds 1.8 mM, which is less than that of seawater (2.2 mM) (D. Yellowlees, unpublished results). However, because the pH of the haemolymph is around 7.3, 0.9 units less than seawater (Fitt *et al.* 1995), the CO_2 concentration of the haemolymph is higher than that of seawater. This results in an outwardly directed CO_2 flux during the night.

The exact location of CA in the gills of marine organisms is an important consideration in determining its physiological role. In organisms where the excretion of CO_2 occurs, for example the crab gill, CA is localized not adjacent to the seawater channels but in the basal epithelium of the gill where it is in contact with haemolymph (Burnett & McMahon 1985). Such a position is

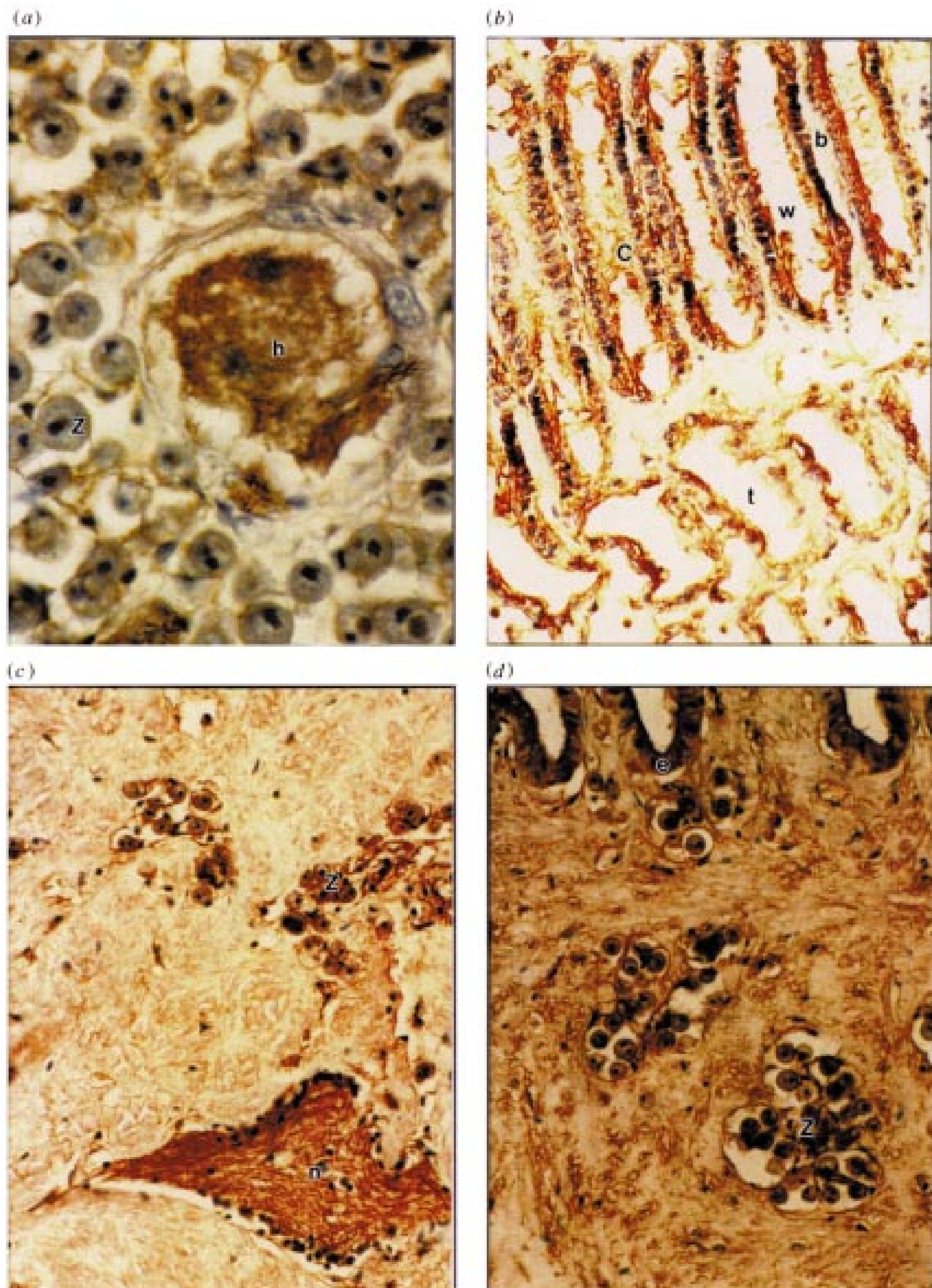

Figure 6. Immunolocalization of CA within the tissues of *Tridacna gigas* using polyclonal antibodies raised against the 70 kDa CA from *T. gigas* gill. (*a*) Mantle (section through the hyaline organ; scale: 10 μm). (*b*) Gill (section through the tertiary water channels and the base of the ciliated branchial filaments; scale: 20 μm). (*c*) Inner siphonal mantle (section showing nerve tissue and zooxanthellae; scale: 20 μm). (*d*) Inner fold of the siphonal mantle (section showing positively staining columnar epithelium; scale: 20 μm). (Key to symbols: b, branchial filament; c, cilia; e, epithelium; n, nerve; t, tertiary water channel; w, water groove; z, zooxanthellae; h, hyaline organ.)

ideal for facilitating the excretion of CO_2 through the gills into the surrounding seawater. The evidence reported here for clams is consistent with a role in facilitating the transport of CO_2 into the clam haemolymph during the day. During the night, however, physiological conditions appear to support the excretion of CO_2 as the haemolymph pH is lower than that of seawater. This will displace the CO_2/HCO_3^- equilibrium, causing CO_2 to

diffuse from the haemolymph into the surrounding seawater.

Immunolocalization of CA also reveals the 70 kDa protein is present on the columnar epithelium which covers the mantle. Here it may well fulfil a similar function to that of the gill CA in facilitating the supply of C_i to the zooxanthellae. Indeed, the mantle pathway may be a significant route for C_i supply to zooxanthellae in the tertiary Z-tubules. The haemolymph is not reticulated within the mantle but is pumped by the heart into the haemal sinuses, which in turn is drained by the venous system to the gills (Norton & Jones 1992). This has implications for the supply of C_i through the gill and, because the Z-tubules abut the mantle surface, the epithelium CA could function to improve the supply of seawater-derived C_i to the algal symbionts.

The 70 kDa CA is also expressed in the hyaline body of the mantle. These are the light-sensing organs ('eyes') of the clam and are typically surrounded on the surface of the mantle by iridophores which appear as iridescent blue–green circles (Norton & Jones 1992). The presence of CA here and in nerve tissue is not unexpected because CAII is also found in high concentrations in the retinal neurons of vertebrates and has been shown to influence retina glial electrical activity (Linser & Cohen 1991). It is also interesting to note that CAII is the isoform that, from our limited amino acid sequence, has the greatest homology with the 70 kDa isoform.

We have previously reported that some clams contain patches of mantle which are bleached (Norton *et al*. 1995) due to atrophy of the Z-tubule system and have few iridophores scattered throughout the bleached areas. Yellowlees *et al*. (1993) inferred a connection between the virtual absence of CA in these patches and the supply of C_i to zooxanthellae. The above results clearly indicate that this is not the whole story. The lack of CA activity in these bleached patches must partly be due to the loss of functional hyaline bodies and presumably the absence of CA from the mantle epithelium. The lack of CA in the bleached patches clearly indicates that the 32 kDa CA is also missing. If there is a CA associated with the Z-tubules, and therefore the supply of C_i to the zooxanthellae, it must therefore be the 32 kDa isoform. A CA of similar mass has been implicated in symbioses between zooxanthellae and cnidarians (Weis *et al*. 1989; Weis 1991). We are currently conducting experiments to determine whether this is the case in tridacnids.

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