Carbonic Anhydrase Expression and Synthesis in the Sea Anemone *Anthopleura elegantissima* Are Enhanced by the Presence of Dinoflagellate Symbionts

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ABSTRACT

Endosymbiotic dinoflagellates resident within cnidarian hosts are extremely productive primary producers. This high productivity may be due in part to an inorganic carbon transport system, present in host tissue, that accelerates carbon delivery to the algae. The enzyme carbonic anhydrase (CA; EC 4.2.1.1) has been shown to be important in this transport system in a variety of tropical symbiotic cnidarians. This study extends the examination of CA to a temperate anemone, *Anthopleura elegantissima*, and documents symbiosis-enhanced production of CA at the biochemical and molecular level. Depending on light availability, *A. elegantissima* can occur naturally with (symbiotic) or without (aposymbiotic) dinoflagellates, making it an ideal study organism for symbiosis-enhanced gene expression. We compared (1) CA activities, (2) quantities of CA using an anti-human CA immunoprobe, and (3) quantities of transcript using a semiquantitative PCR in symbiotic versus aposymbiotic *A. elegantissima* host tissue. Amounts of activity, enzyme, and transcript were greatly enhanced in symbiotic animals compared with aposymbiotic animals. This is the first direct evidence that the presence of symbionts affects the expression of a host cnidarian gene. In addition, we report a full-length *A. elegantissima* CA cDNA sequence, obtained from subcloned reverse transcriptase-PCR products, and its relatedness to α-CAs from a variety of other metazoa, including higher vertebrates.

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Introduction

Symbiotic dinoflagellates resident within endodermal cells of many marine cnidarians are major primary producers in both tropical and temperate communities. The symbiotic algae exhibit rates of photosynthesis comparable to those of free-living macro- and microalgae. There is growing evidence in tropical symbiotic cnidarians that the hosts have acquired mechanisms to accelerate inorganic carbon transport from the seawater pool through host tissues and across several animal and algal membranes, allowing the algae to be highly productive despite their distance from a seawater carbon source (Weis et al. 1989; Weis 1991; Al-Moghrabi et al. 1996; Goiran et al. 1996; Furla et al. 1998*a*, 1998*b*). The enzyme carbonic anhydrase (CA; EC 4.2.1.1), which catalyzes the interconversion of CO_2 and HCO_3^-, is a key component to this CO_2-supply mechanism. CA activity is high in host tissues harboring symbiotic algae (Weis et al. 1989) and can be induced by the presence of algae, as shown during the onset of symbiosis in *Aiptasia pulchella* (Weis 1991). Inhibition of CA activity results in a significant decrease in algal photosynthesis (Weis et al. 1989; Weis 1993). Indeed, CA is thought to be an important component in inorganic carbon transport not only in cnidarian/algal symbioses but also in other marine mutualisms involving autotrophic symbionts, including both the association between the giant clam *Tridacna* spp. and its dinoflagellate symbionts (Yellowlees et al. 1993; Baillie and Yellowlees 1998) and that between various deep sea invertebrates and their sulfur-oxidizing chemosynthetic bacteria (Kochevar et al. 1993; Kochevar and Childress 1996).

In this study, we extend our examination of CA in cnidarians to the temperate rocky intertidal sea anemone *Anthopleura elegantissima*, found on the North American Pacific coast. Animals occurring in sun-exposed locations harbor the dinoflagellate *Symbiodinium californium* (Banaszak et al. 1993) in their endodermal cells (symbiotic animals), whereas animals found in caves or rock crevices lack symbiotic algae (aposymbiotic animals). The facultative nature of the symbiosis in *A. elegantissima* makes it an ideal organism for the study of symbiosis-specific gene expression and protein synthesis. We have previously documented dramatic differences in the protein profiles in symbiotic and aposymbiotic animals (Weis and Levine 1996). These profiles revealed numerous proteins that are unique or enhanced in symbiotic anemones. One abundant symbiosis-

enhanced protein had an apparent molecular weight and isoelectric point identical to CA.

In this article, we detail the differential occurrence of CA in symbiotic versus aposymbiotic *A. elegantissima*, at both the biochemical and molecular level. This is the first direct evidence in a cnidarian/algal association that the presence of symbionts affects the expression of a host gene. In addition, we report a full-length cDNA sequence of CA that has high sequence identity to α-CAs from other metazoa, including vertebrates.

Material and Methods

Animal Collection

Specimens of *Anthopleura elegantissima* (Brandt) were collected near Hopkins Marine Station, Pacific Grove, California. Aposymbiotic animals, which appear bright white in color, were collected from the intertidal zone, underneath the pilings of the neighboring Monterey Bay Aquarium. Light levels in this deep shade environment were as low as 10 μmol quanta/m^2/s (Weis and Levine 1996). Symbiotic animals, which appear brown in color, were collected from neighboring sun-exposed rocks. Small pebbles and shells, which typically adorn *A. elegantissima* columns, were removed by gentle rubbing with gloved fingers. Animals were quick-frozen in liquid nitrogen and shipped via overnight express to Oregon State University on dry ice.

CA Assay

A. elegantissima, with oral disc diameters of approximately 2 cm and wet weights of approximately 1–2 g, were homogenized in a ground-glass tissue grinder in 2 mL of an extraction buffer composed of 25 mM veronal, 5 mM ethylenediaminetetraacetate (EDTA), 5 mM dithiothreitol (DTT), and 10 mM $MgSO_4$, pH 8.2. The resulting crude extract was centrifuged for 10 min at 5,000 *g* at 4°C in order to pellet the unbroken algal cells and chunks of unground animal tissue. The supernatant, which contained animal extract, was decanted and used in the assay.

The in vitro assay for CA activity is described in detail by Weis et al. (1989) and is a modification of the standard pH assay used in many CA studies (for review see Henry [1991]). CA activity was determined by measuring the decrease in pH resulting from the hydration of CO_2 to HCO_3^- and H^+ after addition of substrate. The decrease in pH of the constantly stirred assay mixture was recorded by a Corning Ag/AgCl pH probe connected to a Corning meter fitted with a Linear 1200 chart recorder. As a control for nonspecific change in pH, the same procedure was carried out using animal homogenate that was boiled for 5 min and then cooled to 2°C. CA activity of native and heat-denatured homogenates was measured in triplicate for each sample. Protein-specific activities (ΔpH/min/mg protein) were calculated based on protein assays for each sample (Bradford 1976). These units were chosen to allow comparisons with previous studies (Weis et al. 1989). It is possible, however, to estimate standard enzyme activities expressed in micromoles of CO_2 converted to HCO_3^- and H^+/min/mg protein by multiplying ΔpH/min/mg protein by 0.351 (Kochevar et al. 1993).

Preparation of Animal Protein Homogenates and Polyacrylamide Gel Electrophoresis

The preparation of animal protein homogenates from *A. elegantissima*, and resolution of the resulting proteins using one- and two-dimensional polyacrylamide gel electrophoresis (PAGE), is described in detail by Weis and Levine (1996). Protein homogenates have been shown to be free of algal protein contamination by immunoscreening for algal-specific proteins (Weis and Levine 1996). Animal homogenates were resolved in one dimension using 12.5% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS; Laemmli 1970). Two-dimensional PAGE was performed on the Multiphor II System from Pharmacia, using precast gels, according to the manufacturers instructions.

Immunoblots

For immunoblotting, proteins were electrophoretically transferred from unstained gels, both 1D and 2D, onto nitrocellulose membranes using Pharmacia's Novablot semidry transfer according to the manufacturer's instructions. Immunoblots were developed with a chemiluminescence detection system (Renaissance Kit, Dupont NEN Research Products), and all incubations were carried out on a rotating table. For 1D blots, membranes were first blocked for 1 h at 37°C in 20 mM Tris, 0.5 M NaCl, 0.1% Tween 20, pH 7.5 (TTBS), containing 5% powdered milk. Following this, they were incubated in a 1 : 10,000 dilution of primary antiserum, a polyclonal rabbit anti-human carbonic anhydrase (East Acres Biologicals) in TTBS for 1 h at room temperature, rinsed in 20 mM Tris, 0.5 M NaCl, pH 7.5 (TBS), and subsequently incubated in a 1 : 5,000 dilution of secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (Renaissance Kit, NEN), in TBS. Following a rinse in TBS, the immunoblots were developed according to the manufacturer's directions. For 2D blots, all conditions were the same except membranes were incubated in a 1 : 1,000 dilution of the primary antibody and a 1 : 1,000 dilution of the secondary antibody.

Partial Purification of CA and Protein Microsequencing

Since the putative CA protein made up a significant portion of the protein homogenate from anemones, we could successfully partially purify it from other proteins in a few biochemical steps with relative ease. The presence of the putative CA was monitored through the purification scheme using SDS-PAGE

Table 1: Sequences of oligonucleotide primers used to amplify CA cDNA from *Anthopleura elegantissima*

Primer Name	Nucleotide Position	Nucleotide Sequence and, Where Appropriate, Corresponding Amino Acid Sequence
1-rev (antisense) ……	Downstream of 1040	5′-CTCTAGAACTAGTCTTTTTTTTTTTTTTTTT-3′
2-for ……	142–164	5′-GA(C, T) TT(C, T) CC(C, T) GC(C, T) GC(C, T) GC(C, T) GG(C, T) GC-3′ D F P A A A G A
3-for ……	169–188	5′-CA(A, G) (A, T)(C, G)I CCI AT(A, C, T) GA(G, T) AT(A, C, T) AA-3′ Q S P I D I K
4-for ……	Upstream of 1	5′-TGTCGACGAATTCCCCCCCCCCCC-3′
5-rev (antisense) ……	984–965	5′-AAAGCCTTCATCTCGGCTAC-3′ (noncoding region)
6-rev (antisense) ……	683–664	5′-CC ACT GTA GGT CCA GTA GTC-3′ G S Y T W Y D
7-for ……	441–460	5′-G CTT CAT CTT GTT CAC TGG A-3′ L H L V H W

and looking for a strong 31-kD band. Twenty-five anemone oral discs were homogenized in a Teflon-glass tissue grinder in 20 mL of a 50 mM Tris, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride buffer, pH 7.1. The homogenate was centrifuged for 10 min at 12,000 *g* at 4°C. Ammonium sulfate was added to the supernatant to a final concentration of 65%. The homogenate was gently tipped at 4°C for 1 h and then centrifuged for 30 min at 12,000 *g* at 4°C. The resulting supernatant was dialyzed overnight at 4°C into 10 mM sodium phosphate, 10 mM EDTA, pH 6.5. A 1.5 × 45 cm Superdex 75 size exclusion column (Pharmacia) with a flow rate of 0.6 mL/min was loaded with 700 μL of the sample, containing 2.4 mg of protein. One-milliliter fractions were collected with a fraction collector. Fractions 79–85, containing a total of 162 μg of protein, were pooled and concentrated on a Centricon 10 microconcentrator (Amicon) to a final volume of 100 μL.

In attempting to sequence the CA with automated Edman degradation, we determined that the amino terminus was blocked. The sample was resolved on a 12% SDS-PAGE mini gel. The 31-kD band was excised and subjected to an in-gel LysC digestion (Courchesne et al. 1997). The digest was separated on a Hewlett-Packard 1090 LC using a C18 YMC column (Patterson et al. 1996). Single peaks were subjected to Edman sequencing using a PE-ABD Procise 494 sequencer (Patterson et al. 1996).

RNA Isolation and cDNA Synthesis

Total RNA was isolated from frozen *A. elegantissima* host tissue using methods modified from Chomczynski and Sacchi (1987). Care was taken to remove unbroken algal cells during the extraction procedure to minimize algal RNA contamination (Weis et al. 1998). Anemone tissue was finely minced with a razor blade and then homogenized in a ground-glass tissue homogenizer. We determined empirically that grinding anemones in the denaturing solution consisting of 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% N-lauryl sarcosine, and 100 mM β mercaptoethanol, resulted in algal lysis. Therefore, we homogenized animals in a 1 : 2 dilution of the denaturing solution and a 100 mM sodium phosphate buffer, pH 7.4, containing 450 mM NaCl and 10 mM vanadyl ribonuclease complexes (Life Technologies), a set of compounds that specifically inhibits RNAses. Algae in this homogenization solution remained unbroken, as determined by inspection under a compound microscope. Homogenates were immediately centrifuged in 15-mL sterile polypropylene tubes for 5 min at 7,000 *g* to pellet unbroken algae and unground animal material. The supernatant was decanted and diluted 1 : 2 with denaturing solution. From this stage, samples were processed through the Chomczynski and Sacchi protocol without modification. RNA concentration was determined spectrophotometrically.

Total RNA (5 μg) was reverse transcribed in a 20-μL reaction using 0.5 μg oligo$(dT)_{12–18}$ and 200 U SuperScript II RNase H^- reverse transcriptase (Life Technologies), according to the manufacturer's instructions. The resulting cDNA was then treated with 2 U RNase H to remove the RNA template.

PCR Amplification and Cloning of CA cDNA

Degenerate forward primers, 2-for and 3-for (Table 1; Fig. 1), were designed based on the CA peptide sequence from *A. elegantissima* (see Fig. 3). These primers, together with a reverse oligo (dT) primer, 1-rev, were used in the initial PCR reactions to amplify and clone the 3′ end of the CA cDNA. All PCR and sequencing for this portion of the study was performed on a

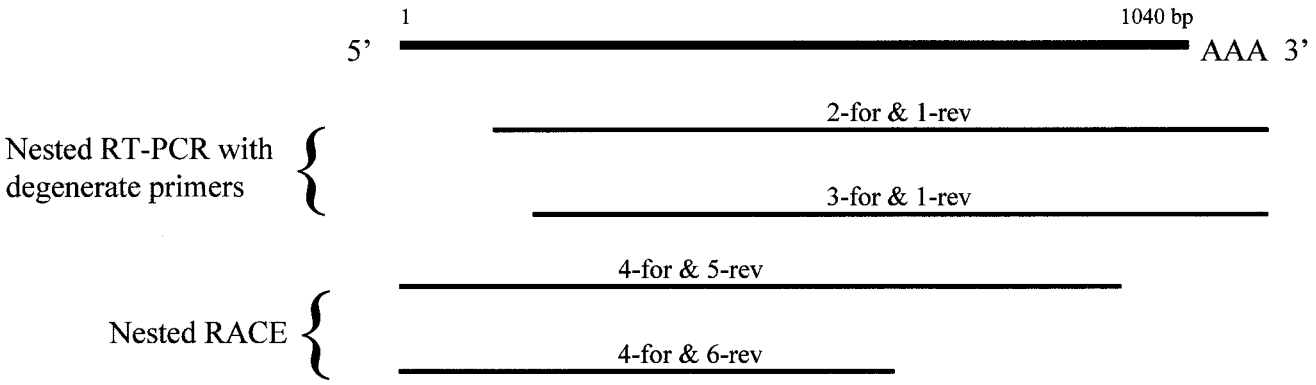

Figure 1. Strategy for amplification and sequencing of complete *Anthopleura elegantissima* CA cDNA. The target CA mRNA, with its poly-A tail, is depicted as the top thick line. The second two lines represent nested PCR products, amplified by RT-PCR and degenerate primers designed from *A. elegantissima* peptide sequences. The last two lines represent nested PCR products generated by 5′-RACE of 5′ poly G-tailed cDNA using an oligo (dC) primer and CA-specific primers. The complete cDNA sequence was obtained by sequencing the second nested product from each set of reactions.

single pool of RNA extracted from multiple anemones from the same clone.

An initial amplification was performed in a 25-μL reaction containing 1 μL of cDNA, 4 μM primer 1-rev, 4 μM primer 2-for, 1.5 U Taq DNA Polymerase (Promega), 2.0 mM $MgCl_2$, and a reaction buffer consisting of 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, and 0.2 mM dNTPs. Reaction conditions were as follows: an initial denaturation step at 94°C for 3 min, followed by three cycles of 94°C (60 s), 37°C (120 s), and 72°C (120 s), then 29 cycles of 92°C (60 s), 60°C (60 s), 72°C (60 s), and a final extension at 72°C for 5 min.

A second PCR amplification was then performed using the internal (nested) CA-specific degenerate primer, 3-for. One microliter of a 1 : 100 dilution of the first PCR product was used as template in a 25-μL reaction containing 0.5 μM primer 1-rev and 2 μM primer 3-for. Reactions were cycled 28 times using an annealing temperature of 56°C.

Two microliters of the nested PCR reaction were ligated into the pCR 2.1 plasmid vector (Invitrogen), according to the manufacturer's instructions, and subsequently used to transform competent INVαF′ *Escherichia coli.*

5′ Rapid Amplification of cDNA Ends (5′-RACE)

In order to amplify the CA sequence upstream from the position of the peptide sequence described above, we employed rapid amplification of 5′ cDNA ends (5′-RACE), modified from Frohman et al. (1988). This included addition of multiple dGTPs to the 5′ end of the cDNA (G-tailing), followed by PCR using an oligo (dC) forward primer, 4-for (Table 1; Fig. 1), and two nested reverse primers, 5-rev and 6-rev (Table 1; Fig. 1).

Total RNA was reverse transcribed as described above except that 2.5 pmol of primer 5-rev (Fig. 1; Table 1) was substituted for oligo (dT). Following reverse transcription, the reaction was treated with 0.4 μg RNase A and 2 U RNase H. The cDNA was purified and then multiple dGTPs were added to the 5′ end using terminal deoxynucleotidyl-transferase (Life Technologies).

After purification, 7.5% of the G-tailed cDNA was used in an initial PCR reaction containing 0.625 μM primer 4-for and 1.25 μM primer 5-rev. Reactions were cycled 39 times using an annealing temperature of 61°C. The sample was resolved on an agarose gel, and a band of the appropriate length, approximately 1,000 bp, was extracted from the gel.

The nested PCR amplification was then performed using 2.5% of the gel extraction product, 0.25 μM primer 4-for, and 0.25 μM primer 6-rev. Reactions were cycled 29 times using an annealing temperature of 62°C. The product of that reaction (0.6 μL) was ligated into the pCR 2.1 plasmid vector (Invitrogen) and subsequently used to transform competent INVαF′ *E. coli.*

Sequencing

Plasmid DNA from individual colonies was purified using a standard small-scale protocol (Sambrook et al. 1989). The plasmid DNA was then sequenced using the Applied Biosystems Taq DyeDeoxy Terminator Cycle Sequencing Kit, and the reaction product was analyzed on an Applied Biosystems model 373 DNA Sequencer. A single contiguous sequence was generated using the Staden Package software.

Semiquantitative PCR

In order to compare CA expression in symbiotic versus aposymbiotic *A. elegantissima*, semiquantitative PCR was per-

formed with CA primers using methods modified from Towle et al. (1997). Semiquantitative PCR techniques vary, but all employ a combination of limiting template, low cycle numbers, and enhanced detection techniques to produce products whose quantities reflect the absolute amount of cDNA present as template. To control for the potential of differential RNA degradation and cDNA synthesis efficiency, we also amplified actin, a presumed housekeeping gene, which should have approximately equal expression regardless of symbiotic state.

Total RNA from three aposymbiotic and three symbiotic animals was extracted as described above and then treated with RNase-free DNase (Ambion), phenol-chloroform extracted, and precipitated with isopropanol. After resuspension in water treated with diethyl pyrocarbonate (DEPC), RNA concentration was determined for each sample spectrophotometrically. Total RNA (2 μg) was reverse transcribed as described above. Duplicate samples were treated under the same conditions except that no reverse transcriptase was added to the samples. CA and actin fragments were then amplified in a single 50-μL reaction containing 5% of the cDNA, 1 μM of each primer: 7-for, 5-rev (Table 1), actin sense primer (5′-TCCCTTGAGAAGAGCTACGA-3′), actin antisense primer (5′-AATGGATGGGCCGGACTCAT-3′), and dNTPs having 1% of the dTTP replaced with digoxigenin-11-dUTP (Boehringer Mannheim). Reaction conditions were: an initial incubation at 94°C for 3 min, followed by cycles of 94°C (45 s), 56°C (60 s), and 72°C (50 s). After 13 and 16 cycles, 9 μL of the reaction were removed. The remaining reaction volumes were cycled 20 times.

Five microliters of the samples were run on a 1.5% agarose gel, blotted onto positively charged nylon membrane (Boehringer Mannheim) overnight, and detected with antidigoxigenin-alkaline phosphatase, Fab fragments, and the chemiluminescence substrate CSPD (Tropix).

Data Bank Searches and Sequence Alignments

Related amino acid sequences were revealed by searching the GenBank database using the BLAST algorithm (Altschul et al. 1990). The predicted anemone CA amino acid sequence was aligned with other CA sequences using MacDNASIS Pro V3.6 (Hitachi Software). The Higgins-Sharp algorithm was used, and minimal adjustments were made by sight. Percent identities between anemone CA and other CAs were calculated using the maximum matching function of DNASIS (Needleman-Wunsch algorithm).

Results

CA Activity and Immunoblots

CA activity in symbiotic *Anthopleaura elegantissima* was high, with an average value of 10.3 $\pm$ 2.8 ΔpH/min/mg protein ($N = 5$), over three times higher than aposymbiotic anemones, which averaged 2.9 $\pm$ 0.4 ΔpH/min/mg protein ($N = 5$). These activities were significantly different from one another, using a two-tailed t-test ($P < 0.001$). This differential pattern was repeated in both 1D and 2D gels and anti-CA immunoblots of symbiotic and aposymbiotic anemone protein homogenates (Fig. 2). Coomassie stained gels revealed a prominent 31-kD band in symbiotic homogenates but a much weaker band in aposymbiotic samples (Fig. 2*A*). On 1D anti-CA blots, symbiotic anemone homogenate had this same strong band at 31 kD when compared with a weaker one in aposymbiotic homogenates (Fig. 2*A*). Two-dimensional blots of symbiotic anemone protein also contained a strongly cross-reactive spot with a pI of 6.3 and molecular mass of 31 kD, whereas the aposymbiotic spot of similar pI and molecular mass was present but much smaller (Fig. 2*B*).

Amplification of CA Sequence using Degenerate Primers, Reverse Transcriptase PCR (RT-PCR), and 5′-RACE

We partially purified CA from symbiotic anemone homogenate and obtained, by protein microsequencing, two peptide sequences; GYGPNEG and DFAAAGARQSPIDIK. The latter sequence aligned strongly with CAs from other organisms, based on BLAST searches (data not shown). From the latter sequence,

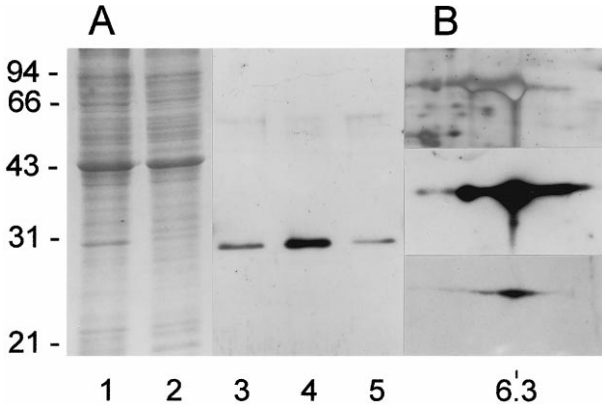

Figure 2. One- and two-dimensional SDS-PAGE and anti-CA immunoblots comparing homogenates of aposymbiotic and symbiotic *Anthopleura elegantissima.* 1D gels and immunoblots are shown in *A.* Molecular weights, determined from a standards lane, are shown on the left in kD. Lanes of anemone tissue contained 20 μg of protein. Lanes 1 and 2 are Coomassie-stained symbiotic and aposymbiotic anemone homogenates, respectively. Lanes 3–5 are anti-human CA immunoblots of bovine CA (1.0 ng loaded) and symbiotic and aposymbiotic anemone homogenates, respectively, showing differential labeling of a band at 31 kD. 2D gels and immunoblots are shown in *B.* All gels contained 30 μg of protein. The top panel is a portion of a silver-stained symbiotic anemone homogenate showing a very strongly staining spot at 31 kD and a 6.3 pI. The middle and bottom panels are anti-CA immunoblots of symbiotic and aposymbiotic anemone homogenates, respectively, showing differential labeling of this spot at 31 kD, pI 6.3.

we designed degenerate primers and used RT-PCR and 5′-RACE to obtain the entire CA cDNA sequence from *A. elegantissima*. The sequencing strategy and accompanying primers are shown in Figure 1 and Table 1, respectively. We designed a degenerate forward primer, named 2-forward (2-for) that, in combination with an oligo (dT) reverse primer, 1-reverse (1-rev), amplified several products using *A. elegantissima* cDNA as template. A second, nested primer (3-for), with oligo (dT), amplified a single discrete band when the first PCR reaction was used as template.

We used 5′ RACE to obtain *A. elegantissima* CA sequence upstream of the described peptide sequence. Another series of nested PCR reactions were performed using G-tailed anemone cDNA as template, with an oligo (dC) forward primer, 4-for, and nested reverse primers, 5-rev and 6-rev, resulting in 1,000 and 700 bp products, respectively.

The complete *A. elegantissima* CA sequence consists of a 1040-nucleotide cDNA with a 783-nucleotide open reading frame (Fig. 3). Three separate 5′ and 3′ end cDNA clones were sequenced. Where discrepancies occurred between the three sequences, we report the most common sequence. The likely start codon is 85 nucleotides downstream from the 5′ end. A poly-A signal, AATAAA, occurs 153 nucleotides downstream from the in-frame stop codon and 13 bases upstream of the poly-A tail itself. The deduced amino acid sequence for *A. elegantissima* CA, 261 amino acids in length, is also shown in Figure 3, as are the locations of the peptide sequences obtained from partially purified anemone CA.

Semiquantitative PCR

To determine whether CA is differentially expressed as a function of symbiotic state in *A. elegantissima*, we performed semiquantitative RT-PCR of anemone cDNA using specific primers for CA (7-for and 5-rev; Table 1). Actin, a presumed housekeeping gene, was also amplified to control for differential RNA degradation and cDNA synthesis efficiency. These results are shown in Figure 4. From all samples, the CA primers amplified a band that corresponded closely to the predicted size of 543 based on the cDNA sequence. The actin primers amplified a 400 bp product in all samples. Bands amplified using the CA primers were much darker in the three symbiotic compared with the aposymbiotic anemone samples. In contrast, bands amplified using the actin primers were of approximately equal intensity in all anemone cDNAs. Control PCR reactions using untranscribed samples as template produced no visible bands (data not shown), indicating that the bands described above originated from transcribed RNA and not contaminating DNA.

Alignments of Anemone CA with CA from Other Animals

BLAST searches of GenBank using the anemone sequence revealed dozens of alignments with α-CAs (data not shown). An alignment of the *A. elegantissima* CA-deduced amino acid sequence with a subset of these sequences is shown in Figure 5. These are *Danio rerio* (zebrafish) CAH (NIH accession U55177), human CA I (X05014) and CA II (Y0039, J03037), *Drosophila melanogaster* CAH (L39622, as translated by Hewett-Emmett and Tashian [1996]), and *Caenorhabditis elegans* CA1 (U12966, as modified by Hewett-Emmett and Tashian [1996]). Sequences were chosen both to represent CAs from organisms across broad phylogenetic distances within the animal kingdom (nematodes through higher vertebrates) and to show a varying degree of sequence identity. *A. elegantissima* CA was 47% identical to human CA I, 46% to human CA II, 50% to *D. rerio* CAH, 42% to *D. melanogaster* CA, and 35% identical to *C. elegans* CA1. The highest homologies in all cases occurred in regions surrounding the active site of the functional enzyme, specifically the histidine-binding sites for the zinc cofactor (shown by squares) and the associated hydrogen bond network (shown by asterisks; Sheridan and Allen 1981; Hewett-Emmett et al. 1984; Fukuzawa et al. 1990; Fukuzawa and Ishida 1991).

Discussion

This study extends the examination of CA in symbiotic cnidarians to a temperate anemone species, demonstrates differential enzyme and transcript levels as a function of the symbiotic state, and describes a cDNA sequence that shares considerable homology with α-CAs from a wide variety of other metazoans.

Enhanced Synthesis of CA in Symbiotic Anthopleura elegantissima

The pattern previously observed in tropical cnidarians of high CA activity in symbiotic animals compared with nonsymbiotic animals (Weis et al. 1989) is strongly confirmed in this temperate species of anemone. Indeed, the symbiotic *Anthopleura elegantissima* homogenates had the highest CA activities that we have measured for cnidarians. The average value of 10.3 ΔpH/min/mg protein is approximately 40% higher than the highest values we have previously reported from an anemone, *Condylactis gigantea*, a gorgonian *Gorgonia ventalina*, and a zoanthid, *Palythoa variabilis*, all approximately 7.0 ΔpH/min/mg protein (Weis et al. 1989). We have previously contended that CA activity is negatively correlated with the surface area-to-volume ratio of the cnidarian host, with highly branched or platelike morphologies having low CA activities and fleshy, massive morphologies having high CA activities (Weis et al. 1989; Lesser et al. 1994). Small surface areas and increased diffusion distances presumably slow inorganic carbon transport and therefore increase the need for CA to aid in the transport

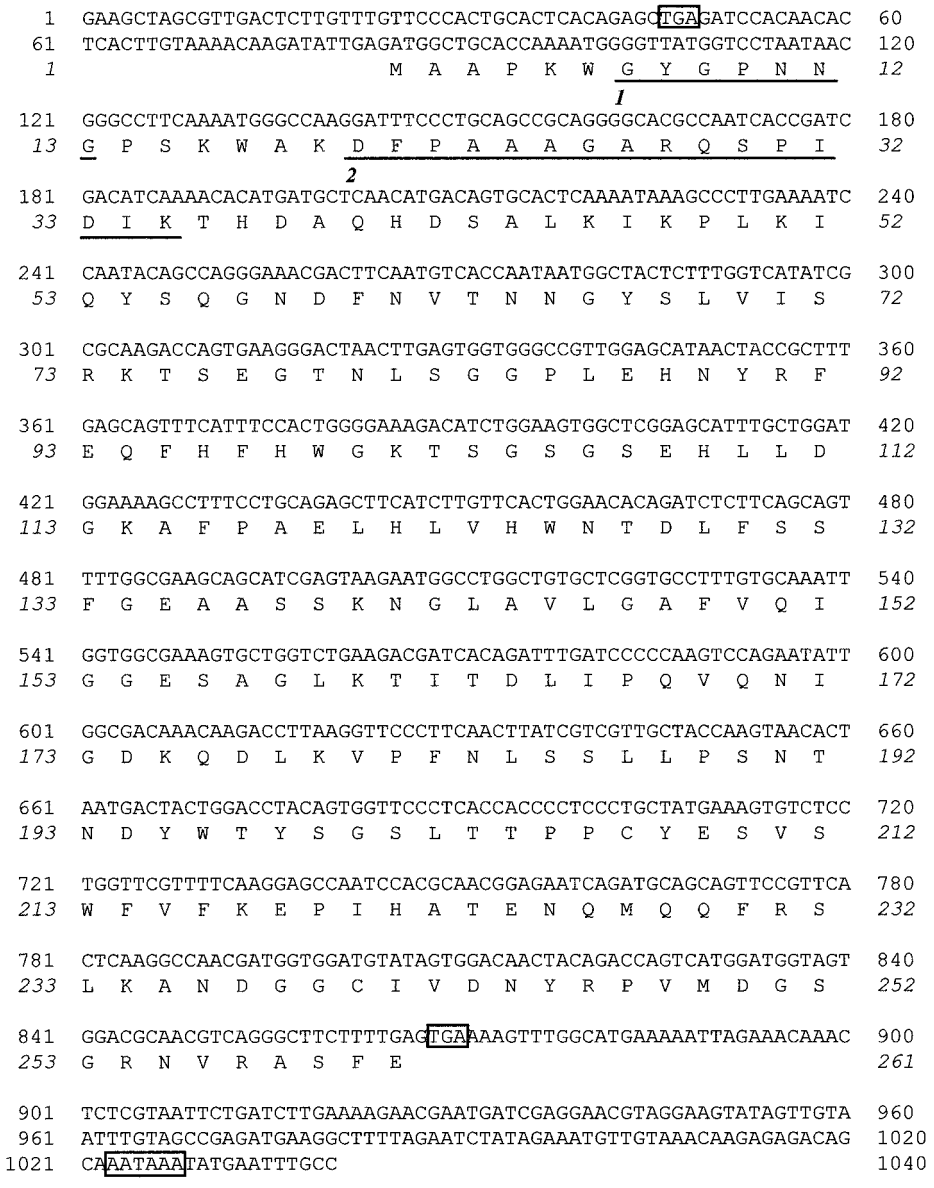

Figure 3. Nucleotide sequence and predicted amino acid sequence of *Anthopleura elegantissima* CA. The sequence is a 1040-nucleotide cDNA with a 783-nucleotide open reading frame. The deduced amino acid sequence is 261 amino acids in length. The nucleotide and amino acid numbers are shown on the sides of the sequence. Stop codons, TGA, present in the sequence are boxed, one occurring in the 5′ untranslated region and another occurring at the end of the open reading frame. The polyadenylation signal, AATAAA, which occurs 13 bases upstream of the beginning of the poly-A tail, is also boxed. The locations of the two peptide sequences obtained from partially purified *A. elegantissima* CA are shown as underlines. The peptide sequence numbered 2 was the sequence used in designing degenerate oligonucleotide primers for RT-PCR. GenBank accession number AF140537.

process. *A. elegantissima*, with its short, fleshy column, short tentacles, and therefore low surface area-to-volume ratio, coupled with extremely high rates of CA activity, fits well with this trend.

Immunoblots using an anti-human CA labeled bands on 1D gels at 31 kD and spots on 2D gels at 31 kD, 6.3 pI, biochemical characteristics very similar to those of the α-CAs (Wyeth and Prince 1977). Strong cross-reactivity between a polyclonal anti-vertebrate CA and an *A. elegantissima* protein suggests that this protein is a highly conserved member of the CA gene family. We also performed 1D immunoblots using a polyclonal antibody generated from a CA purified from the nonsymbiotic

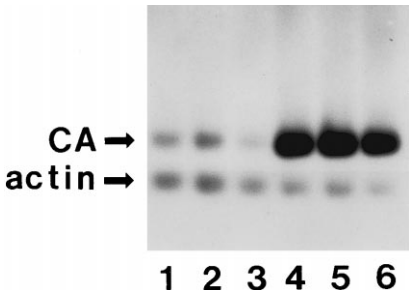

Figure 4. Estimation of CA and actin mRNA levels in aposymbiotic and symbiotic *Anthopleura elegantissima* using semiquantitative RT-PCR. Following reverse transcription of RNA, CA and actin fragments were amplified from the cDNA in a single reaction. Lanes 1–3 are products from aposymbiotic anemone RNA, and lanes 4–6 are products from symbiotic anemone RNA. The upper 550 bp fragment was generated using CA primers 7-for and 6-rev (see Table 1) and was amplified much more strongly from symbiotic RNA than from aposymbiotic RNA. The lower 400-bp fragment was generated using actin primers and was approximately equally amplified from all samples, regardless of symbiotic state.

calcifying gorgonian coral *Leptogorgia virgulata* (Lucas and Knapp 1996). This antibody also labeled a 31-kD band in *A. elegantissima* homogenates (data not shown) and further suggests the presence of a CA in anemone homogenates.

The immunoblots using anti-human CA showed much stronger spots and bands in symbiotic host homogenates than in aposymbiotic ones, a pattern identical to what we have described previously in the tropical anemone *Aiptasia pulchella* (Weis 1991). This is consistent with the differential CA activities observed between the two anemone types. These data suggest that high CA activity present in symbiotic animals is caused by the presence of high amounts of the enzyme in symbiotic animals relative to aposymbiotic ones, as opposed to the two animal types possessing similar enzyme amounts but with differential activities. Immunolocalization experiments determining the cellular location of this putative CA in both symbiotic and aposymbiotic animals are an obvious next step to further understanding its role in host as well as symbiont physiology.

Enhanced Expression of CA in Symbiotic A. elegantissima

As with CA activities and immunological analyses, host CA transcript levels increased with the presence of symbiotic algae in host tissue, as shown using semiquantitative PCR. These data extend our previous results on differential occurrence of CA to the level of the gene. To our knowledge, this is the first study in cnidarian/algal associations that describes the up-regulation of a gene as a function of symbiosis. Future genetic studies may elucidate the manner in which the symbiotic state dictates the regulation of CA expression in anemones.

Alignments of Anemone CA with α-CA from Other Animals

The evolution of CA gene families has recently been rigorously reviewed by Hewett-Emmett and Tashian (1996). CA has evolved independently at least three times and exists in extant groups as α-, β-, and γ-CA: the α-CAs occurring in animals and some plants and eubacteria, the β-CAs occurring in plants and eubacteria, and the γ-CAs occurring in eubacteria and archaebacteria. Of the dozens of matches we obtained with α-CAs using BLAST searches, we show alignments of *A. elegantissima* CA with those from several other animals displaying varying degrees of sequence identity (Fig. 5). Anemone CA aligns more closely with zebrafish CA and several mammalian isoforms, specifically CAs I, II, III, V, and VII (data for III, V, and VII not shown), than with *Drosophila melanogaster* and *Caenorhabditis elegans* CAs and other mammalian isoforms, including CAs IV, VI, VIII, and IX (data not shown). In areas surrounding the active site that binds the zinc cofactor, sequence identity nears 100% with vertebrate CAs (Fig. 5). Since *A. elegantissima* CA is the only lower metazoan CA described to date, its sequence will make a contribution to our understanding of CA evolution. Detailed phylogenetic analyses, such as those carried out by Hewett-Emmett and Tashian (1996), however, are beyond the scope of this study.

CA as a "Symbiosis Gene"

This study corroborates previous work on the importance of CA in cnidarian/algal associations. With an immunological probe for the protein and nucleic acid probes for the RNA well in hand, studies of CA in cnidarian/algal symbiosis can move in two directions. First, regulation of CA: experimental studies can focus on what interpartner signaling events, such as changes in intracellular pH or translocation products, take place to initiate CA synthesis and expression. Second, onset of symbiosis: CA can be used as a specific marker for the symbiotic state in studies of symbiosis onset between cnidarians and their dinoflagellate symbionts. So little is known about the events surrounding the initiation of the partnership that the ability to track the onset of expression of a "symbiosis gene" could give valuable information on both the time course and the nature of these early molecular interactions between the partners.

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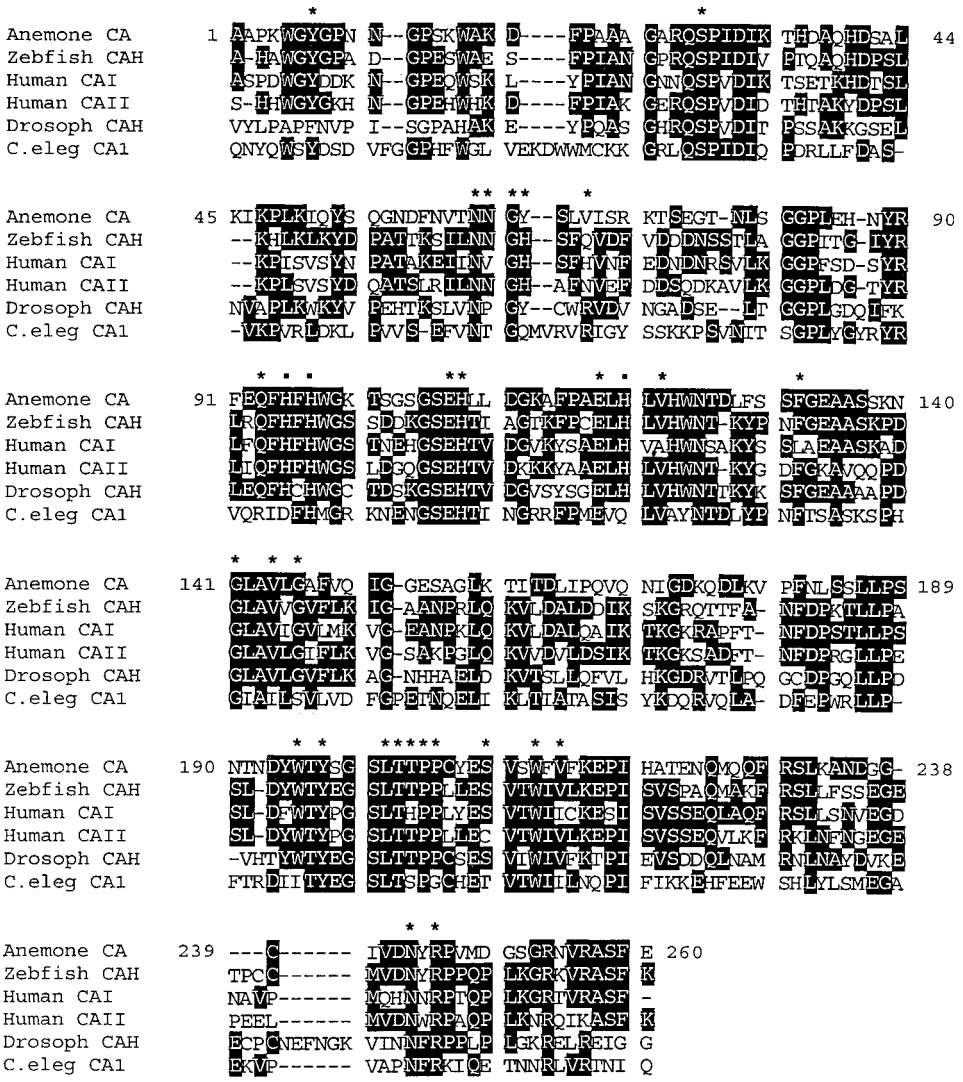

Figure 5. Alignment of *Anthopleura elegantissima* predicted amino acid sequence and α-CAs from *Danio rerio* (zebrafish) CAH, human CAI and CAII, *Drosophila melanogaster* CAH, and *Caenorhabditis elegans* CA1, based on analysis by MacDNASIS software. Numbering is based on the *A. elegantissima* sequence. Regions of conserved amino acids are shown in black. Squares mark histidines that directly bind the zinc cofactor, and asterisks represent amino acids participating in the hydrogen bond network surrounding the active site (Sheridan and Allen 1981; Hewett-Emmett et al. 1984; Fukuzawa et al. 1990; Fukuzawa and Ishida 1991).

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