Involvement of H\textsuperscript{+}-ATPase and carbonic anhydrase in inorganic carbon uptake for endosymbiotic photosynthesis

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Furla, Paola, Denis Allemand, and Maria-Novella Orsenigo. Involvement of H\textsuperscript{+}-ATPase and carbonic anhydrase in inorganic carbon uptake for endosymbiotic photosynthesis. Am J Physiol Regulatory Integrative Comp Physiol 278: R870–R881, 2000.—Symbiotic cnidarians absorb inorganic carbon from seawater to supply intracellular dinoflagellates with CO\textsubscript{2} for their photosynthesis. To determine the mechanism of inorganic carbon transport by animal cells, we used plasma membrane vesicles prepared from ectodermal cells isolated from tentacles of the sea anemone, Anemonia viridis. H\textsuperscript{14}CO\textsubscript{3}\textsuperscript{−} uptake in the presence of an outward NaCl gradient or inward H\textsuperscript{+} gradient, showed no evidence for a Cl\textsuperscript{−} or H\textsuperscript{+}-driven HCO\textsubscript{3}\textsuperscript{−} transport. H\textsuperscript{14}CO\textsubscript{3}\textsuperscript{−} and Cl\textsuperscript{−} uptakes were stimulated by a positive inside-membrane diffusion potential, suggesting the presence of HCO\textsubscript{3}\textsuperscript{−} and Cl\textsuperscript{−} conductances. A carbonic anhydrase (CA) activity was measured on plasma membrane (4%) and in the cytoplasm of the ectodermal cells (96%) and was sensitive to acetazolamide (IC\textsubscript{50} = 20 nM) and ethoxyzolamide (IC\textsubscript{50} = 2.5 nM). A strong DIDS-sensitive H\textsuperscript{+}-ATPase activity was observed (IC\textsubscript{50} = 14 μM). This activity was also highly sensitive to vanadate and allyl isothiocyanate, two inhibitors of P-type H\textsuperscript{+}-ATPases. Present data suggest that HCO\textsubscript{3}\textsuperscript{−} absorption by ectodermal cells is carried out by H\textsuperscript{+} secretion by H\textsuperscript{+}-ATPase, resulting in the formation of carbonic acid in the surrounding seawater, which is quickly dehydrated into CO\textsubscript{2} by a membrane-bound CA. CO\textsubscript{2} then diffuses passively into the cell where it is hydrated in HCO\textsubscript{3}\textsuperscript{−} by a cytosolic CA.

symbiosis; anthozoan; sea anemone; HCO\textsubscript{3}\textsuperscript{−} transport; carbon-concentrating mechanism

HCO\textsubscript{3}\textsuperscript{−} transporting epithelia play a major role in the acid-base balance of all organisms. The transepithelial HCO\textsubscript{3}\textsuperscript{−} transport is maintained either by secretion or reabsorption of the ion from the luminal medium to the body fluids. This leads to a net flux of acid or base across the epithelial barrier without net accumulation of inorganic carbon inside the epithelium (13). The oral epithelial layers of symbiotic Anthozoa were recently found to absorb HCO\textsubscript{3}\textsuperscript{−} from the surrounding seawater to supply algal endosymbionts with CO\textsubscript{2} for their photosynthesis (5, 34, 25; see review, Ref. 4). However, in contrast to other HCO\textsubscript{3}\textsuperscript{−} transporting epithelia, this transport system in oral layers of Anthozoa leads to a concentration of inorganic carbon inside the epithelium itself. This mechanism is well known in algal and cyanobacterial cells (8), but, in animal cells, has only been described in hydrothermal invertebrates (33).

The body of Anthozoa resembles a bag with three-layered walls. Two cellular layers, the ectoderm (facing the environment) and the endoderm (facing the digestive cavity called coelenteron), are separated by a cell-free connective layer, the mesoglea. Endodermal cells contain symbiotic phototroph Dinoflagellates, generally known as zooxanthellae. Therefore, dissolved inorganic carbon (DIC) has to cross several animal membranes to become available for the photosynthetic fixation by the CO\textsubscript{2}-fixing enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) within the zooxanthellae. We previously demonstrated that DIC is absorbed from the seawater by the ectodermal cell layer and then transferred to the endodermal cell layer. In the latter, HCO\textsubscript{3}\textsuperscript{−} is dehydrated to CO\textsubscript{2} and used for symbiont photosynthesis and OH\textsuperscript{−}, which is secreted within the coelenteric cavity (11, 25). This process leads to a functional polarization of the oral layers, the endodermal face being alkaline (25). The major supply of DIC then results from a transcellular transport of HCO\textsubscript{3}\textsuperscript{−} (25), whereas a fraction (<20%) is supplied by passive diffusion (26).

The mechanism of HCO\textsubscript{3}\textsuperscript{−} absorption by the anthozoan ectodermal cells remains unknown. In sea anemones, the Na\textsuperscript{+} insensitivity of symbiotic O\textsubscript{2} production rules out the involvement of Na\textsuperscript{+}-dependent HCO\textsubscript{3}\textsuperscript{−} uptake (11), as well as Na\textsuperscript{+}/H\textsuperscript{+} exchange-mediated HCO\textsubscript{3}\textsuperscript{−} dehydration (25). Other hypotheses include Cl\textsuperscript{−}-HCO\textsubscript{3}\textsuperscript{−} symports, H\textsuperscript{+}-HCO\textsubscript{3}\textsuperscript{−} cotransport, membrane-associated carbonic anhydrase (CA), or H\textsuperscript{+}-ATPase-mediated HCO\textsubscript{3}\textsuperscript{−} dehydration (4). In this paper we propose to characterize the apical mechanism(s) of HCO\textsubscript{3}\textsuperscript{−} uptake by the ectodermal cells of Anthozoa. For this purpose we used an apical plasma membrane-enriched fraction of ectodermal cells from sea anemone tentacles following the method previously described by Bajorat and Schlichter (10).

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MATERIALS AND METHODS

Biological Material

Specimens of the Mediterranean sea anemone Anemonia viridis (Forskal) were collected in Villefranche-sur-mer, France, and maintained in an open-circuit seawater aquarium supplied with Mediterranean seawater pumped at a depth of 50 m. Light was provided by a metal halide lamp (HPI-TS 400 W, Philips), with a photosynthetic photon flux density of 125 umol m⁻² s⁻¹ and a 12:12:12 photoperiod.

Preparation of Brush-Border Membranes Vesicles

Brush-border membrane (BBM) vesicles (BBMV) from A. viridis tentacles were prepared by a magnesium aggregation and differential centrifugation technique (temperature between 0 and 4°C) according to Bajorat and Schlichter (10) with some modifications. Briefly, 3–4 g of fresh tentacle tissue was homogenized in 30 ml sorbitol buffer [in mM: 10 sorbitol, 2 Tris-HCl pH 7.1, 0.2 phenylmethylsulfonyl fluoride (PMSF), 1 1,4-dithiothreitol (DTT), 0.01% ethanol] using an Ultra Turrax T25 (20,000 rpm) for 90 s. After filtration through a 250-µm pore size nylon mesh, MgCl₂ was added to the homogenate (final concentration of 10 mM) and stirred occasionally in an ice bath for 15 min. MgCl₂ aggregates preferentially all membranes except brush border (55). After centrifugation of the homogenate at 3,000 g for 15 min with a Centrikon T-1080 and rotor SW41 (Kontron), the supernatant was collected and centrifuged at 27,000 g for 30 min. Two other steps of membrane aggregation by magnesium and centrifugation were necessary to obtain optimal purification. For the uptake experiments, the last pellet was suspended in the experimental medium and centrifuged at 30,000 g for 20 min.

For CA measurements, the endodermal cells were scraped under a binocular microscope using forceps (12) to separate them from the ectodermal cell layer. Homogenate, cytoplasm, and membrane vesicle preparations of ectodermal or endodermal cells were then used.

Histological Studies

The recovered vesicle fraction was resuspended in a 90 mM phosphate buffer, pH 7.4, containing 250 mM sorbitol and centrifuged at 30,000 g for 20 min. The pellet was fixed for 1 h at 4°C in 2.5% glutaraldehyde in 90 mM phosphate buffer, pH 7.4, followed by five 10-min washes in the same buffer. Subsequently, the samples were postfixed in 1% osmium tetroxide in 90 mM phosphate buffer for 1 h at 20°C, rinsed twice in distilled water, and dehydrated in 30, 50, 70, 80, and 100% ethanol. Samples were infiltrated with Epon 812 for 4 h at 20°C, subsequently embedded in capped gelatin capsules with fresh Epon 812 and allowed to polymerize for 2 days at 60°C in a vacuum oven. Ultrathin sections (60 nm) were cut with a diamond knife using a Reichert Jung Ultracut ultramicrotome and stained with uranyl acetate (6 min, 7% in methanol) and lead citrate (6 min). After being rinsed in distilled water, the sections were observed with a CM 12 Phillips Transmission Electron Microscope at 100–120 kV.

Uptake Experiments

The transport experiments involving 8–9 mM [³⁵Cl⁻]NaCl (specific activity 1.21 × 10¹⁰ Bq/mol; ICN) or 2–3 mM [¹⁴C] NaHCO₃ (specific activity 0.19 × 10¹² Bq/mol; NEN, Boston, MA) were carried out in triplicate at 25°C using a rapid filtration technique (48). A volume of BBM suspension (4–8 mg protein/ml) was mixed with the proper incubation solution. The compositions of the resuspension buffers and incubation media are detailed in Figs. 2–5 legends. All resuspension buffers contained valinomycin, an ionophore shown to be effective on plasma membrane of A. viridis (15). Stock solution of valinomycin was prepared in ethanol (2.5 mM). An aliquot of the stock solution (5 µl/100 µl of BBMV suspension) was introduced in the Eppendorf tube to obtain a final concentration of 125 µM. Before the addition of BBMVs, the alcohol was evaporated and valinomycin remained adherent on the Eppendorf tube. Samples were collected at selected times and diluted with 1 ml ice-cold reaction stop solution [402 mM potassium gluconate for H¹⁴CO₃⁻ uptake in presence of 400 mM chloride gradient or 139 mM potassium gluconate for other uptake experiments, 0.2 mM PMSF, 0.01% (vol/vol) ethanol, 20 mM HEPES-Tris buffer at pH 7.0, 8.2, or 9.0 according to the experiment]. The samples were then filtered on cellulose nitrate filters (0.45 µm pore size) and rinsed with 5 ml of the stop solution. Radioactive filters were counted in a scintillation counter (Packard 1600 Tricarb) after addition of 4 ml Aqualuma Plus. In the technique of membrane vesicle purification, intravesicular space varies between each preparation, inducing differences in absolute H¹⁴CO₃⁻ or ³⁵Cl⁻ uptake results. For that reason, although uptake experiments were performed at least three times with different membrane vesicle preparations and gave qualitatively identical results, we chose to present, only in figures showing experiments of isotopic uptakes (Figs. 2–4), representative values of a single experiment. Each point of the single experiment was obtained by three replicates (represented by means ± SE).

Assay of Marker Enzymes

The degree of purification of the recovered fractions relative to the initial homogenate was evaluated by determining the enrichment factors of various marker enzyme according to Bajorat and Schlichter (10): alkaline phosphatase (BBM marker) according to Walter and Schutt (59), γ-glutamyl transferase (BBM marker) according to Persijn and van der Slik (52), total and Na⁺–K⁺-activated ATPase (basolateral membrane marker) according to Gratecos et al. (31), and cytochrome-c oxidase (mitochondrial marker) according to Cooperstein and Lazarruva (19). Each measurement was made in triplicate, with at least three different preparations of vesicles.

Assay of CA and ATPase Activity

CA activity was determined according to Maren (44). One unit of enzyme activity (U) was equal to (T₀ – T)/T, where T₀ and T represent the reaction times for the uncatalyzed and the catalyzed reaction, respectively.

H¹⁻, Cl⁻⁻, and HCO₃⁻-dependent ATPase activities were assayed colorimetrically using a microplaque reader (Lab-systems Multiskan Bichromatic) at 37°C, by measuring the release of P₃ from ATP according to Mayer-Gostan and Lemaire (45). Eight micrograms protein for the homogenate, 6 mM MgCl₂, 20 mM Tris-HEPES pH 7 and 2 mM ATP-Tris; for HCO₃⁻-ATPase and Cl⁻-ATPase assay, 200 mM NaHCO₃ or NaCl, respectively, 6 mM MgCl₂, 20 mM Tris-HEPES pH 7 and 2 mM ATP-Tris. Preliminary experiments were performed to determine the optimal concentration of Mg²⁺ in the reaction mixture: 10 mM MgCl₂ was chosen for H⁻-ATPase activity assay and 6 mM MgCl₂ for HCO₃⁻ or Cl⁻-ATPase activity assays (results not shown). In some experiments, freshly isolated zoanthellae (F12), obtained as previously described.
zyme, and cytochrome-c

tion (Fig. 1) showed the presence of closed vesicles
changed. Electron micrographs of the recovered frac-
tions of 25 and 50 mM, respectively. For the
measurement of
amino)-benzoate (NPPB) and diphenylamine-2-carboxylate
(DPC), were dissolved in 100% ethanol to obtain stock solutions of
25 and 50 mM, respectively.

Purification of Ectodermal BBMV

RESULTS

Pharmacology

DIDS, an anion carrier inhibitor, was dissolved in the
proper experimental solution to obtain a stock solution of
10 mM. The anion channel inhibitors, 5-nitro-2(3 phenylpropyl-
aminio)-benzoate (NPPB) and diphenylamine-2-carboxylate
(DPC), were dissolved in 100% ethanol to obtain stock solutions of
25 and 50 mM, respectively. For the measurement of
CA activity, the CA inhibitors acetazolamide (AZ) and ethoxy-
zaamide (EZ) were dissolved in the reaction mixture. For the
ATPase characterization, AZ was dissolved in 1 mM Tris-
buffered DMSO (pH 8.2).

Stock solution of the H+-ATPase inhibitors: oligomycin,
bafilomycin, and phenylglyoxal (PGO) stock solutions were
prepared in DMSO at 10 mM, 100 µM, and 500 mM,
respectively; diethylstilbestrol (DES), N,N’-dicyclohexylcarbo-
dimide (DCCD), and N-ethylmaleimide (NEM) were pre-
pared in ethanol at 12, 40, and 50 mM, respectively; and allyl
isothiocyanate and sodium azide were prepared in distilled
water at 1 and 100 mM, respectively. Sodium orthovanadate
(10 mM was dissolved in hot water at 100 µM, and the pH
was adjusted to 7.2 with HCl. To obtain the ATPase-inhibiting
forms (H2VO4 and HVO42), the stock solution was subjected to
four successive cycles of heating and cooling, adjusting the pH
to 7.2 with NaOH for each cycle.

A stock solution of EGTA was prepared in distilled water,
adjusting pH to 6 with NaOH. Sodium thiocyanate (SCN-)
dissolved in distilled water at 1 M.

Although preliminary experiments demonstrated that a
concentration of DMSO, or ethanol, up to 2% (vol/vol) causes
no significant effect on flux studies, CA, or ATPase activities
(results not shown), we used these solvents at a concentra-
tion ≤0.5%.

All chemicals were obtained from Sigma or Merck, except
NPPB, which was obtained from Tocris and DPC, a generous
gift from Dr. M. Avella (Nice Sophia-Antipolis University,
France).

Presentation of Results

Results were standardized according to the protein content
measured in the homogenate and the vesicle fraction. Protein
concentrations were quantified by the method of Bradford
(14) using a spectrophotometer Kontron Unikon 931 and
bovine serum albumin as a standard. In multiple treatment
groups, the results were validated by one-way ANOVA and
Bonferroni-Dunn post hoc tests, whereas Student’s t-test was
used when comparing two different groups. Results were
considered statistically significant when P < 0.05.

RESULTS

Purification of Ectodermal BBMV

To determine the origin of the membrane vesicles
obtained from A. viridis tentacles, enrichment factors
of marker enzymes in the collected fraction were mea-
sured (Table 1). Alkaline phosphatase and γ-glutamyl
transferase, two well-known marker enzymes of the
BBM, were enriched in the recovered fraction, whereas
Na+-K+-ATPase, a basolateral membrane marker en-
zyme, and cytochrome-c oxidase, a mitochondrial
marker enzyme, were either reduced or almost un-
changed. Electron micrographs of the recovered frac-
tion (Fig. 1) showed the presence of closed vesicles

(diameter ranging from 0.1 to 0.6 µm) and a very weak
contamination with other subcellular structures. Some
vesicles were still filled with electron dense material,
which presumably originated from the core of the
microvilli (55).

Uptake Experiments

The first set of experiments was performed by using
rapid filtration technique to test the possible presence
of ion symports or antiports involved in apical mem-
brane uptake of HCO3 in ectodermal cells of A. viridis.
The filters (0.45 µm pore size) used for these experi-
ments did not retain all vesicles but allowed an optimal
rate of filtration. Consequently, the resulting uptake
values were underestimated.

Effect of medium osmolarity on 36Cl uptake.

Figure 2 shows that, after 40 min of vesicle incubation, the
equilibrium uptake of 8–9 mM 36Cl− decreased by a
linear relationship (r2 = 0.9795) with increasing osmo-
licity of the extravesicular medium. This result con-
firms the presence of closed vesicles in the recovered
fraction used for uptake experiments. The extrapolation
of Cl− uptake to infinite osmolarity showed the absence
of significant chloride binding on the surface of the
vesicles (chloride binding at zero osmolarity = 0.15
nmol·mg protein−1·min−1).

Effect of membrane potential on chloride and bicarbon-
ate uptake.

Figure 3 shows the effect of positive inside-
membrane diffusion potential on Cl− and HCO3− up-
take. The enhancement of Cl− and HCO3− uptake in the
presence of a +116 mV membrane potential created by
imposing inwardly directed 124.74 mM potassium gra-

![Fig. 1. Electron microscopic study of brush-border plasma mem-
brane (BBM) vesicles of Anemone viridis. A: view of a collection of
intact vesicles. B: enlargement of BBM vesicles.](image-url)
in the presence of valinomycin, strongly suggesting the presence of a conductive pathway for these two anions in the BBMs. However, although the Cl\(^-\) conductance was insensitive to 400 µM DIDS (11), the HCO\(_3^-\) conductance was sensitive to this inhibitor (Fig. 3, A and B).

Figure 3, C and D, illustrates the dependence of 3-min incubation Cl\(^-\) and HCO\(_3^-\) uptake on membrane potential ranging from 0 to +120 mV. Figure 3C shows that Cl\(^-\) conductance was independent on membrane potential up to +60 mV and linearly dependent beyond this value. The HCO\(_3^-\) conductance was linearly dependent on the membrane potential beyond +30 mV and only poorly affected below this value (Fig. 3D). No saturation was observed in the potential range tested for the two conductances.

Effect of DIDS, NPPB, and DPC on chloride and bicarbonate uptake. To characterize the Cl\(^-\) and HCO\(_3^-\) conductances, the effects of three anion channel blockers were studied on 3-min uptake. Figure 4 shows that DPC, at a concentration of 500 µM (7), did not affect the two conductances (ANOVA, Bonferroni-Dunn post hoc test; \(P > 0.31\)). However, 100 µM NPPB (57) slightly inhibited chloride conductance, although not significantly (ANOVA, Bonferroni-Dunn post hoc test; \(P > 0.013\)). DIDS (400 µM) inhibited only HCO\(_3^-\) conduc-

![Graph](image1.png)

**Fig. 2.** Effect of extravesicular osmolarity on uptake of 8–9 mM \(^{36}\)Cl\(^-\) after 40 min equilibration. BBM vesicles (45 µl; 5–8 mg protein/ml) were incubated for 40 min with 90 µl of incubation medium. Vesicles were obtained in 70 mM sorbitol and 125 µM valinomycin, and incubation medium contained 8–9 mM \(^{36}\)Cl\(^-\) and 133, 300, 700, and 1000 mM sorbitol. All solutions contained 100 mM potassium gluconate, 20 mM HEPES-Tris buffer at pH 8.2, 0.2 mM phenylmethylsulfonyl (PMSF), 0.01% (vol/vol) ethanol. After 40 min incubation, 90-µl samples were processed as described above. Points reported on curve are means ± SE of 3 replicates of a representative experiment.

![Graph](image2.png)

**Fig. 3.** Uptake experiments on BBM vesicles of *A. viridis*. Effect of membrane potential and 400 µM DIDS on \(^{36}\)Cl\(^-\) uptake (A) and H\(^{14}\)CO\(_3^-\) uptake (B): 27 µl BBM vesicles obtained in 250 mM sorbitol, 1.26 mM potassium gluconate, and 125 µM valinomycin were incubated in 504 µl of either 1.26 mM potassium gluconate and 250 mM sorbitol (A), or 126 mM potassium gluconate (B), or 126 mM potassium gluconate and 400 µM DIDS (C). Dependence of \(^{36}\)Cl\(^-\) uptake (C) and H\(^{14}\)CO\(_3^-\) uptake (D) on positive inside membrane diffusion potential: 8 µl BBM vesicles obtained in 250 mM sorbitol, 1.26 mM potassium gluconate, and 125 µM valinomycin were incubated in 142 µl of incubation medium contained various concentrations of potassium gluconate concentration and sorbitol to obtain different membrane diffusion potential according to intravesicular osmolarity. All solutions contained 20 mM HEPES-Tris buffer at pH 8.2, 0.2 mM PMSF, 0.01% (vol/vol) ethanol. Points reported on curves are means ± SE of 3 replicates of a representative experiment. *Significantly different from values obtained when experiment was performed in absence of DIDS (Student’s t-test; \(P < 0.05\)).
Results are means \pm SE of 3 replicates of 3 different homogenates of A. viridis whole tentacle, ectodermal and endodermal layers presented a similar CA activity (Student’s t-test; P > 0.16). Similarly, a pH unit gradient [intravesicular pH (pHi) = 6; extravesicular pH (pHe) = 7] did not enhance \(^{14}\text{CO}_3\)/uptake (Student’s t-test; P > 0.33). To prevent the dehydration of \(^{14}\text{CO}_3\), this experiment was also carried out in the presence of 600 \(\mu\text{M}\) AZ, and no differences were recorded (results not shown). In conclusion, these results provide no evidence for a \(\text{Cl}^-/\text{HCO}_3^-\) exchanger or an \(\text{H}^+/-\text{HCO}_3^-\) cotransport in apical ectodermal plasma membrane of A. viridis.

Effect of proton gradient on \(^{36}\text{Cl}^-\) uptake. \(^{36}\text{Cl}^-\) uptake, in the presence of a pH gradient (pH \(_i\), 9; pH \(_o\), 7) was measured to determine the possible presence of a \(\text{Cl}^-/\text{OH}^-\) exchanger (or \(\text{Cl}^-/-\text{H}^+\) cotransport). Incorporation of \(^{36}\text{Cl}^-\) in the lumen of vesicles after 3 min of incubation was the same with or without pH gradient, thus showing the absence of a \(\text{Cl}^-/\text{OH}^-\) exchanger (or \(\text{Cl}^-/-\text{H}^+\) cotransport) in apical membrane of ectodermal cell (control: 2.201 \(\pm\) 0.047 nmol \cdot \text{mg protein}^{-1} \cdot 3\text{ min}^{-1}; change in pH = 2: 2.856 \(\pm\) 0.266 nmol \cdot \text{mg protein}^{-1} \cdot 3\text{ min}^{-1}; Student’s t-test; P > 0.22).

CA Assay

Previous results (11) suggested that DIC absorption by A. viridis tentacle is partly dependent on CA activity. Measurement of CA activity was then performed on cell homogenates (whole tentacle or ectodermal or endodermal cells), apical plasma membrane of ectodermal or endodermal cells, and FIZ. Table 3 shows that both cell layers presented a similar CA activity (Student’s t-test, P > 0.05), whereas that of FIZ was 3.5-fold lower. To determine whether animal CA activities were cytoplasmic or associated to the plasma membrane, specific and total CA activities were measured in both total and plasma membrane-enriched fractions. The results, summarized in Table 4, show that >95% of the total cellular CA activity in both cell layers was located in the cytosolic fraction of both cell layers.

To characterize the cytoplasmic CA activity of ectodermal and endodermal layers, a dose-response curve with AZ and EZ, two sulfonamide inhibitors, was performed (Fig. 5). The inhibition constants (IC\(_{50}\)) for ectodermal CA were 21.15 \(\pm\) 2.61 \(\mu\text{M}\) for AZ and 3.33 \(\pm\) 0.59 \(\mu\text{M}\) for EZ, whereas those of endodermal CA were 21.98 \(\pm\) 0.38 and 4.54 \(\pm\) 0.58 \(\mu\text{M}\), respectively. The effect of DIDS on CA activity was also tested. DIDS concentrations up to 125 \(\mu\text{M}\) did not affect significantly the activity of CA in whole tentacle homogenates (results not shown),
Table 4. Carbonic anhydrase specific and total activities in homogenates and BBM vesicles of A. viridis ectodermal cell layer and endodermal cell layer

<table>
<thead>
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<th>Specific Activity, U/mg protein</th>
<th>Total Activity (U), % of yield</th>
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<tbody>
<tr>
<td>Ectodermal cell layer</td>
<td></td>
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<tr>
<td>Homogenate</td>
<td>6.14 ± 0.32</td>
<td>133 ± 20 (100%)</td>
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<tr>
<td>BBM vesicles</td>
<td>12.58 ± 0.20</td>
<td>5.0 ± 0.1 (3.8 ± 0.1%)</td>
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<tr>
<td>Endodermal cell layer</td>
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<tr>
<td>Homogenate</td>
<td>5.31 ± 0.22</td>
<td>113 ± 6 (100%)</td>
</tr>
<tr>
<td>BBM vesicles</td>
<td>13.25 ± 0.37</td>
<td>4.0 ± 0.1 (3.6 ± 0.1%)</td>
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Results are means of enzyme units (U) ± SE of at least n = 3–5 homogenate or plasma membrane vesicle preparations.

whereas 400 µM DIDS, a concentration that inhibits ~40% of DIC transport (11), inhibited CA activity by ~17% (control CA activity in total homogenate: 4.93 ± 0.21 U/mg protein; CA activity in presence of 400 µM DIDS: 3.78 ± 0.25 U/mg protein).

**ATPase Assays**

Measurement of H⁺-ATPase activity. Presence of an H⁺-ATPase in corals (5) and in sea anemones (11, 25) has been suggested. To confirm the presence of this pump in A. viridis tissues, H⁺-ATPase activity was characterized in apical plasma membrane vesicles of ectodermal cells. To evaluate any possible contamination of the preparation by intracellular H⁺-ATPases, the sensitivity to inhibitors for different classes of ATPases was tested (Table 5). Oligomycin, a known inhibitor of mitochondrial ATPase, had little effect on ATP hydrolysis even at high concentrations (Fig. 6D).

Azide, another mitochondrial ATPase inhibitor, inhibited ATP hydrolysis of BBMVs only at high concentrations (1 mM produced 40% of inhibition; IC₅₀ = 610 µM, Fig. 6C). On the other hand, ATP hydrolysis was almost totally inhibited by micromolar concentrations of orthovanadate (IC₅₀ = 10 µM; Fig. 6A), a known inhibitor of P-type ATPases (24), and by 100 µM DIDS (IC₅₀ = 14 µM; Fig. 6B), a common anion carrier inhibitor (16). In both cases, the vesicle fraction was more sensitive than total homogenate (Table 5). Allyl isothiocyanate, another P-ATPase inhibitor (46), inhibited ~50% of vesicle ATPase activity in the micromolar range. Vacuolar H⁺-ATPase inhibitors, bafilomycin (1 µM) and NEM (500 µM), had no effect on homogenate and vesicle ATPase activities. DES and DCCD, two wide-spectrum H⁺-ATPase inhibitors, were used. DES (100 µM) inhab-
ited ATP hydrolysis, whereas DCCD (400 µM) had no effect (Table 5).

Other inhibitors were also tested. PGO (5 mM), a blocker of H\(^+\)-NO\(_3\) symport, inhibited 42% of ATP hydrolysis in the vesicle fraction and showed no effect on homogenate. ATP hydrolysis was insensitive to 600 µM AZ, a CA inhibitor, and to the presence of K\(^+\) and Ca\(^{2+}\).

To determine the target of the different inhibitors used, we measured the effect of azide in addition to other inhibitors. The effect was additive for almost all inhibitors tested, including oligomycin (Table 5). This suggests that these inhibitors act on different targets and confirms that the major part of the ATPase activity measured in the vesicle fraction is of nonmitochondrial origin. Vanadate was the only inhibitor that did not have an additive effect with azide, suggesting that azide and vanadate act on the same target.

Measurement of Cl\(^{-}\)-ATPase activity. Controversial literature exists on plasma membrane-bound anion-stimulated ATPase (see reviews, Refs. 29, 21). Nevertheless, the work of Gerencser and Lee (30) presents strong evidence that the plasma membrane of enterocytes isolated from the marine mollusk Aplysia contains true Cl\(^{-}\)-ATPase activity.

Measurement of Cl\(^{-}\)-ATPase activity in presence of increasing concentrations of NaCl is shown in Fig. 7. Maximal Cl\(^{-}\)-ATPase activity (4.32 µmol P\(_i\) h\(^{-1}\) mg protein\(^{-1}\)) was achieved at 150 mM Cl\(^{-}\), and the apparent Michaelis constant (K\(_m\)) for Cl\(^{-}\) is 32 mM. Thus 200 mM NaCl was used to further characterize the plasma membrane Cl\(^{-}\)-ATPase of A. viridis. As for H\(^+\)-ATPase activity, the most important inhibition of...
plasma membrane Cl–-ATPase activity was measured with vanadate (1 mM, 68% of inhibition, \( P < 0.025 \)) and DIDS (400 \( \mu \)M, 72% of inhibition, \( P < 0.025 \)). Azide (1 mM), oligomycin (5 \( \mu \)M), and SCN– (10 mM) did not produce any inhibition on the Cl–-ATPase activity of homogenate or vesicles. Oligomycin insensitivity rules out any contamination by a mitochondrial Cl–-ATPase activity. The important effect of vanadate suggests that A. viridis Cl–-stimulated ATPase is a phosphorylated ATPase similar to the one studied in the intestine of Aplysia (30). Moreover, the apparent \( K_m \) for Cl– (32 mM) was in the same range of the Aplysia gut cell Cl– affinity (\( K_m = 10.3 \) mM; Ref. 30). However, Aplysia Cl–-ATPases present high thioscyanate sensitivity, which is in contrast with the present results (30).

Measurement of HCO3–-ATPase activity. Increasing concentration of HCO3– did not enhance ATP hydrolysis (result not shown), suggesting that there is no HCO3–-stimulated ATPase in the apical plasma membrane of A. viridis ectodermal cells.

DISCUSSION

Recent studies have demonstrated that ectodermal cells of anthozoan exhibit active transport mechanisms to absorb bicarbonate from the seawater and supply intracellular symbiont photosynthesis with inorganic carbon (5, 11, 25). Using a purified membrane preparation, we present strong evidence that bicarbonate transport by the apical membrane of ectodermal cells is dependent on a proton pump and a CA.

Nature of Recovered Membrane Vesicles

The use of membrane vesicles to study transport in marine invertebrates is restricted to few models (23, 28, 49). In Anthozoa, this technique has been validated by Bajorat and Schlichter (10). These authors showed that the use of MgCl2 to precipitate basolateral membranes of whole tentacle homogenate allows recovery of only apical BBM of oral ectodermal cells, i.e., microvillosities-bearing membrane facing seawater.

Because of the lack of invertebrate-specific biochemical tools, vertebrate marker enzymes are often used in marine invertebrates (1, 10, 30, 38, 64). In the present study, the most important enrichment factors after purification of membrane vesicles were obtained with alkaline phosphatase and \( \gamma \)-glutamyl transferase, whereas the enrichment in Na+-K+-ATPase was very low. This result is in agreement with an enrichment in apical plasma membrane of recovered vesicles reported by Bajorat and Schlichter (10). Enrichment in cytochrome-c oxidase shows some mitochondrial contamination in the ectodermal plasma membrane vesicles.

Pharmacological studies of H+-ATPase activity in vesicles allowed for estimation of this contamination, taking into account that without knowing the specificity of inhibitors on the cnidarian cell, results must be interpreted with caution. The important sensitivity of vesicle H+-ATPase activity to azide, a mitochondrial (F1F0)-type H+-ATPase inhibitor (46), suggests an important contamination by mitochondrial membranes, but three arguments refute this conclusion: 1) the nonadditive effect with vanadate, a P-type H+-ATPase inhibitor (24), suggesting a common target of these two inhibitors; 2) the little effect of the two other F1F0-ATPase inhibitors, oligomycin and DCCD (even if DCCD is less specific on mitochondrial ATPase than oligomycin (3)); and 3) the additive effects of oligomycin and vanadate. These results suggest that, in sea anemone, azide may inhibit not only F1F0-ATPase, but also other types of ATPases, whereas oligomycin should be more specific to F1F0-ATPase. In conclusion, contamination of recovered vesicles with mitochondrial membranes could be quantified by the extent of inhibition by oligomycin, i.e., ~17% (see Table 5).

Symport and Antiport

Bénazet-Tambutté et al. (11) demonstrated that inorganic carbon uptake by ectodermal cells of the sea anemone is performed by Na+-independent carrier proteins. Allemand et al. (4) suggested that these carriers could be an H+-HCO3– cotransporter or a Cl–/HCO3– exchanger, which have been described in a large range of marine photrophs, including cyanobacteria, microalgae, and macrophytes (review, Ref. 8). Nevertheless, the present data using 38Cl or H14CO3– show no evidence for the presence of Cl–- or H+-dependent HCO3– transport. Only DIDS-sensitive anion conductance was demonstrated. These results rule out the involvement of a secondary active transport of HCO3– in the apical plasma membrane of ectodermal cells. However, an H+-HCO3– cotransport and/or a Cl–/HCO3– exchanger may play a role in basolateral membrane of ectodermal cells, allowing HCO3– efflux toward endodermal cells. This hypothesis could explain the external DIDS effect (an anion exchanger inhibitor) on photosynthesis (11, 25).

CA

The role of CA in HCO3–-transporting epithelia is well documented in vertebrates (37). Its involvement in photosynthetic processes is well described in marine algae and cyanobacteria, where it is assumed that CA plays a major role in carbon-concentrating mechanisms (2, 9). The uncatalyzed rate of dehydration of HCO3– into CO2 is a relatively slow process at the pH of seawater (pH 8.2). CA increases this rate at least 1,000-fold (40), allowing rapid equilibration of HCO3– and CO2, which creates an inward gradient of CO2, thereby favoring CO2 absorption.

CA has also been detected in marine symbiotic photrophs (61), where it can play a role in carbon supply to the symbiont photosynthesis (5, 60). In these studies, CA activity has been measured only in homogenate of whole tentacles (60), whereas distribution of the enzyme has been studied by immunocytochemistry (5, 60, 61). Until now, the presence of CA in ectodermal cells has been suggested on the basis of pharmacological experiments (5, 11, 25) and only recently has its activity been measured in the jellyfish Cassiopea xamachana (51). By measuring CA activity in the different cell
layers, our results demonstrate the presence of CA activity in both oral cell layers of the Mediterranean sea anemone. These results differ with the CA immunolocalization shown to be on or near zooxanthellae within endodermal cells (5, 60, 61). These contrasting data show that commercial antibodies against human CA do not allow recognition of all forms of CA and suggest the presence of at least two isoforms: one linked to or induced by the symbionts [as suggested by Weis et al. (61)], localized on perisymbiotic (60) or on algal membrane (5), and one specific to the cnidarian cell. Antibodies recognize only the first type of CA, as suggested by Al-Moghrabi et al. (5).

CA activities are associated with 1) the soluble fraction of the cytosol of both ectodermal and endodermal cells; 2) the BBM of both ectodermal and endodermal cells, and 3) zooxanthellae. In the two types of epithelial cells, the major fraction of CA activity is located within the animal cytoplasm (96%), as previously reported by Weis (60) in the sea anemone Aiptasia pulchella.

Because the intracellular pH is generally more acidic than the pH of seawater, the presence of a CA within the cytoplasm of the host cell can be important: it may either increase or decrease the CO2 leakage according to which carbon species enters the cell. If HCO3 enters the host cell, then CA would promote CO2 leakage by increasing the rate of dehydration of HCO3. On the other hand, when CO2 enters the cell, CA would decrease its leakage by favoring its equilibration with HCO3. The high level of CA activity in the cytoplasm of ectodermal cell argues in favor of a carbon-concentrating mechanism based on primary extracellular dehydration of HCO3 into CO2, followed by passive diffusion of CO2. However, this cannot exclude an important role of plasma membrane-bound CA in HCO3 absorption, as shown in the medullary collecting duct of rabbit (58).

CA specific activities of ectodermal plasma membrane (12.58 ± 0.20 EU/mg protein) and endodermal plasma membrane (13.25 ± 0.37 EU/mg protein) are within the same range as measured in BBMV of eel intestine (19.51 ± 1.21 EU/mg protein; Ref. 43). Only a weak activity is detected in zooxanthellae, as previously reported (35, 61).

Similar effects of AZ and EZ on total ectodermal and endodermal CA activities (Fig. 7) suggest the presence of only one cytoplasmic isozyme of CA in both cell layers. As generally reported in literature (50), cytoplasmic CA activity is more sensitive to EZ (IC50 = 3.33 ± 0.59 and 4.54 ± 0.58 nM, respectively, for ectodermal and endodermal cells; P = 0.174) than AZ (IC50 = 21.15 ± 2.61 and 21.98 ± 0.38 nM; P = 0.701). Our results show that sea anemone CA activity is almost totally insensitive to DIDS. Because previous results (11, 25) showed that photosynthesis is partly sensitive to DIDS, these results demonstrate that CA cannot be the only mechanism of inorganic carbon transport in anthozoan cells.

In conclusion, we suggest that inorganic carbon absorption by the apical membrane of ectodermal cells is carried out by a plasma membrane-bound and by intracellular CA, the first favoring extracellular HCO3 dehydration and thereby CO2 absorption, whereas the cytoplasmic CA would prevent the leakage of CO2 by increasing its rate of equilibration with HCO3. These CA would be different isoforms than the CA associated with the presence of zooxanthellae. CA, however, is not the only one mechanism for DIC uptake.

H+-ATPase

Allemand et al. (4) suggested two putative mechanisms for inorganic carbon absorption: a direct primary transport of bicarbonate or an indirect mechanism implicating a proton pump. Present results do not show any evidence for an HCO3-stimulated ATPase activity, whereas this activity has been described in Aplysia gut (30) using the same technique. However, we found an important DIDS-sensitive H+-ATPase activity.

Three major classes of H+-ATPase have been described in animal cells that differ in their cell localization and in their structural, functional, and pharmacological properties: P-ATPases, V-ATPases, F1F0-ATPases (review, see Ref. 24). P-ATPases are always present in plasma membranes and have a phosphorylated intermediate, which accounts for their vanadate sensitivity. V-ATPases are generally vacuolar but can also be located transiently or permanently in the plasma membrane. F1F0-ATPases are present in animal cells only in the inner membrane of mitochondria. V- and F1F0-ATPases do not present any phosphorylated intermediate.

Our results clearly show an H+-ATPase activity in BBM of oral ectodermal cells. As previously shown (see above), most of the measured H+ pump activity is linked to the plasma membrane and does not result from a contamination by mitochondrial or microsomal membranes. The measured activity (7.08 ± 0.97 µmol P1·h−1·mg protein−1) is comparable with the H+-ATPase activity in plasma membrane of cyanobacterium (5.8 ± 0.3 µmol P1·h−1·mg protein−1; Ref. 27), but is still much lower than the H+-ATPase activity of renal brush-border proximal cell (37.9 ± 2.56 µmol P1·h−1·mg protein−1; Ref. 41).

A. viridis H+-ATPase activity has been characterized by its sensitivity to different inhibitors. However, pharmacological characterization of H+ pump-related ATPase is difficult because of the lack of specific inhibitors. Proton pump activity measured in BBMVs is highly sensitive to vanadate and DIDS and moderately sensitive to allyl isothiocyanate. The important DIDS effect (~100% of inhibition) could account for the great in vivo sensitivity of DIC-dependent O2 evolution in Anthozoa (5, 11, 25). A similar sensitivity of membrane-bound H+-ATPase activity has been reported in a few cases (3, 6, 63), DIDS could react with lysine residues in proteins leading to nonspecific effects (22). In sea anemone, a low concentration of DIDS (IC50 = 14 µM) affects P1 release, suggesting a specific action. Our data do not show any effect of AZ on P1 release. This demonstrates that the effect of AZ observed in vivo (5, 11, 25) is not the result of an inhibition of a proton pump but rather a direct effect on CA activity.
Allyl isothiocyanate and vanadate are generally known to be specific inhibitors of mammalian P-ATPases. Vanadate competes with Pi and prevents the formation of the intermediate phosphoenzyme complex (24, 36, 47). Thus these results suggest that the ectodermal plasma membrane H\(^{+}\)-ATPase is of P-type. However, data show that ATPase activity is independent of the presence of potassium or calcium. This rules out the involvement of an H\(^{+}\)-K\(^{+}\)-ATPase or a Ca\(^{2+}\)-ATPase activity and suggests an electrogenic activity. This is in contrast with a classic animal P-type activity, because no electrogenic P-type plasma membrane H\(^{+}\)-ATPase has been described in animal cells (32), except in the fish gills (42).

We can also suggest that H\(^{+}\)-ATPase activity measured in ectodermal plasma membrane is a vanadate-sensitive V-ATPase similar to the one described by Chatterjee et al. (18) in osteoclast plasma membrane. However, two arguments refute this assumption: the first one is the high vanadate sensitivity of the sea anemone H\(^{+}\)-ATPase (IC\(_{50}\) = 10 µM), typical of animal P-type ATPase inhibition (IC\(_{50}\) are 10-fold higher for V-ATPase; Ref. 18); the second argument is the weak effects of bafilomycin and NEM, two specific mammalian V-type ATPase inhibitors (24, 36, 47).

Other inhibitors present some moderate effect (DES, azide, and phenylglyoxal) or weak effect (DCCD), but their specificity is more uncertain (3, 63). Therefore, no definitive conclusion can be given. We can suggest that H\(^{+}\)-ATPase activity of sea anemone resembles in some way the vanadate-sensitive P-type H\(^{+}\) pump, sharing some similarities to electrogenic P-type plasma membrane H\(^{+}\)-ATPases described in plants (56).

Anion Conductances

H\(^{+}\)-ATPase activity is often associated with passive Cl\(^{-}\) conductance in the vacuolar compartment or in the plasma membrane (24). In the latter case, a proton pump and chloride conductance drive HCl net flux. A Cl\(^{-}\) conductance also allows for the dissipation of the high membrane potential established during ATP-driven H\(^{+}\) transport. Therefore, the Cl\(^{-}\) conductance measured in the apical membrane of ectodermal cells (Fig. 3, A and C) could be linked to the proton pump activity. The efflux of chloride through the ectodermal apical membrane depends on the electrochemical gradient of chloride. The Nernst electrochemical potential of chloride can be theoretically calculated as follows: \(E_{Cl^-} = \frac{-RT}{zF} \log\left(\frac{[Cl^-]_{in}}{[Cl^-]_{out}}\right)\approx -28 \text{ mV}\), where \(R\), \(T\), and \(z\) have their usual thermodynamic meanings (gas constant, absolute temperature, valency of the anion, respectively); the intracellular chloride concentration is \(\sim 200 \text{ mM}\) (54), and the extracellular chloride concentration is \(\sim 600 \text{ mM}\). The membrane potential is unknown, but can be assumed to be in the range of \(-70\) to \(-123 \text{ mV}\), as measured in marine invertebrate cells (17). Consequently, Cl\(^{-}\) efflux through ectodermal cells is passive.

Our results also demonstrate the presence of an HCO\(_3^{-}\) conductance that shares similar sensitivity to the two anion blockers (NPPB and DPC) with the Cl\(^{-}\) conductance but differs in DIDS sensitivity. HCO\(_3^{-}\) conductance is significantly blocked by DIDS, whereas Cl\(^{-}\) conductance is totally insensitive to this inhibitor. This pattern of sensitivity suggests that these conductances are not an artifact resulting from passive leakage of ions. Some differences also appear in the membrane potential dependence of Cl\(^{-}\) and HCO\(_3^{-}\) conductance. For both channels, the anion transports were dependent on membrane potential only up to 40 mV (Fig. 3, C and D). Similar membrane potential dependence was described by De Giorgi et al. (20) in eel intestine. These differences suggest the existence of two different channels.

Although we measured an HCO\(_3^{-}\) conductance in apical membrane of ectodermal cells, we can exclude a direct implication of this channel in apical HCO\(_3^{-}\) absorption, because Nernst electrochemical potential of bicarbonate predicts a passive efflux of this ion (5).

In conclusion, in addition to previous physiological results obtained on whole epithelium (11, 25), present data suggest that HCO\(_3^{-}\) absorption by ectodermal cells...
of the sea anemone for endosymbiont photosynthesis is dependent on a DIDS- and vanadate-sensitive H\(^+\)-ATPase and an AZ- and EZ-sensitive CA. Our results suggest that, in symbiotic anthozoan ectodermal cells, HCO\(_3^-\) is absorbed by a mechanism very similar to that shown in vertebrate kidney. By analogy to the HCO\(_3^-\) absorption in the proximal tubule, we can suggest that HCO\(_3^-\) absorption is carried out by an active H\(^+\)-ATPase-mediated H\(^+\) secretion. This mechanism creates an important hyperpolarization of the cell membrane, with the apical Cl\(^-\) conductance allowing it to dissipate. In contrast to what occurs in kidney, an Na\(^+\)/H\(^+\) exchanger, although present in apical membrane of ectodermal cells, does not play any role in HCO\(_3^-\) absorption in sea anemone (25). The secretion of H\(^+\) results in the formation of carbonic acid in the seawater surrounding the cell, which is dehydrated into CO\(_2\). This step is further enhanced by membrane-bound CA, which are known to be often associated with a plasma membrane H\(^+\) pump (43). CO\(_2\) then diffuses passively into the cell where it is hydrated in HCO\(_3^-\) by a cytosolic CA. HCO\(_3^-\) could exit the basolateral membrane through an electroneutral Cl\(^-\)/HCO\(_3^-\) antiporter (Fig. 8). Our results also suggest the presence of a Cl\(^-\)-ATPase in the plasma membrane of ectodermal cells; its role remains to be determined.

**Perspectives**

Symbiotic anthozoans provide an original and fascinating model for the study of HCO\(_3^-\) transport. In these primitive animals, HCO\(_3^-\) is absorbed by animal cells to supply photosynthetic symbionts with CO\(_2\). Present results demonstrate some similarities with vertebrate systems. However, numerous points need to be examined: what are the mechanisms of inorganic carbon transport from ectodermal to endodermal cells? How is this transport system regulated, because, on photosynthesis-induced cell alkalosis, anthozoan cells stimulate an HCO\(_3^-\)-uptake system known in vertebrates to protect cells from acidosis. This different regulation pathway could be the consequence of the origin of this transport system, which, as previously suggested by Raven (53), could result from gene transfer from the symbiont to the host nucleus.

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