Membrane-associated carbonic anhydrase in gills of the blue crab, *Callinectes sapidus*

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**Henry, Raymond P.** Membrane-associated carbonic anhydrase in gills of the blue crab, *Callinectes sapidus*. Am. J. Physiol. 252 (Regulatory Integrative Comp. Physiol. 21): R966-R971, 1987.—The presence of the enzyme carbonic anhydrase (CA) on the basal membrane of the branchial endothelial cells in the blue crab and its physiological significance were studied in vivo using a membrane-impermeant CA inhibitor, quaternary ammonium sulfanilamide (QAS). Injection of QAS into the hemolymph of *Callinectes sapidus* resulted in the rapid development of a respiratory acidosis; *P*CO₂ rose almost 2 Torr, pH was lowered by ~0.25 units, and total CO₂ rose by 2 mM. These results support the hypothesis that membrane-associated CA exposed to hemolymph is present in the crustacean gill and that it is physiologically significant in mobilizing hemolymph HCO₃⁻ to CO₂ to facilitate CO₂ excretion across the gill. The recovery from this acidosis coincides with the clearance of the inhibitor from the hemolymph. Hemolymph osmotic and ionic parameters were unaffected by QAS, reconfirming the role of branchial cytoplasmic CA in ion regulation and also providing a convenient bioassay for determining CA inhibitor permeability in the intact organism.

**THE CRUSTACEAN GILL** is known to be involved in a number of physiological processes. Primarily the organ of gas exchange (O₂ uptake and CO₂ excretion), the gill, is also important in osmotic and ionic regulation (5, 6, 12, 20, 25) and in maintaining hemolymph acid-base balance (6, 12, 14, 28, 29). The enzyme carbonic anhydrase (CA) is found in high concentrations in the crustacean gill (12, 13). CA catalyzes the reversible hydration of CO₂ to H⁺ and HCO₃⁻. Because the products of the hydration/dehydration reaction are both gaseous and ionic species and can be involved in gas exchange, ion transport, and acid-base regulation, CA has been postulated as being a central enzyme to all three processes in the crustacean gill (12, 13). CA catalyzes the reversible hydration of CO₂ to H⁺ and HCO₃⁻, which serve as counterions for the active uptake of Na⁺ and Cl⁻, respectively, in both crustacean and fish gills (4, 12, 15, 18). There is also evidence suggesting that branchial CA mobilizes hemolymph HCO₃⁻ to CO₂ facilitating CO₂ excretion across the gill (1, 2, 23). Since crustacean hemolymph does not possess CA activity, extrabranchial catalysis of HCO₃⁻ to CO₂ must be excluded (12, 13). The two processes (ion transport and CO₂ excretion) involve net catalysis in the hydration and dehydration directions, respectively, within the gill, and thus they cannot be taking place in the same cellular compartment (i.e., cytoplasm) simultaneously. Also the basal membrane of the gill in fish has been shown to be impermeable to HCO₃⁻ (24), a condition that is likely also to exist in crustaceans; thus cytoplasmic CA does not appear to have access to hemolymph HCO₃⁻ and most likely cannot function in CO₂ excretion.

Branchial CA localization in a subcellular compartment that is accessible to hemolymph HCO₃⁻ could, however, function in CO₂ excretion. Specifically CA associated with the basal membrane of the gill with its active site exposed to the hemolymph would be able to mobilize HCO₃⁻ to CO₂ for excretion.

Other tissue types, involved in both respiration and ion transport, are known to possess membrane-associated CA. The mammalian lung has CA associated with the endothelial membrane and exposed to plasma (7, 8, 16, 19, 30), and the kidney tubule also has CA present on the luminal brush border (17, 22, 31). Outside of the mammals, however, virtually nothing is known about the presence, distribution, or function of membrane-associated CA in respiratory and ion-transporting tissues.

One of the few nonmammalian systems to have received attention has been the crustacean gill in which CA can potentially have multiple functions. Burnett et al. (4) originally stated that there was no extracellular membrane-associated CA in the crustacean gill and that CO₂ excretion was facilitated by intraepithelial CA. Based on experiments using the freely permeable CA inhibitors acetazolamide and ethoxzolamide, which cannot physiologically distinguish membrane-associated CA from cytoplasmic CA, the presence of membrane-associated CA in the crustacean gill has been inferred (1, 2, 23). A more recent study by Burnett and McMahon (3), using a large-molecular weight dextran-bound CA inhibitor (DBI) (27) in an isolated perfused gill preparation, has indicated that there is extracellular CA on the branchial membrane.

The presence and function of crustacean branchial membrane-associated CA has never been directly studied in the intact organism, however. Existing reports on branchial CA function performed on intact animals are contradictory. Burnett et al. (4) and Henry and Cameron (15) reported a nonrespiratory hemolymph alkalosis after treatment with acetazolamide, the opposite effect from...
what would have been expected (i.e., a respiratory aci-
dosis) if branchial membrane-associated CA were present
and functioning in CO2 excretion. McMahon et al. (23)
did, on the other hand, report a respiratory acidosis
caused by acetazolamide. Part of the problem is that
acetazolamide permeates the branchial membrane and
inhibits total gill CA; thus it cannot differentiate between
the putative respiratory role of extracellular membrane-
associate CA and the ion regulatory role of soluble cyto-
plasmic CA in the whole organism. Another CA inhibitor,
quaternary ammonium sulfanilamide (QAS), has been
shown to be an effective inhibitor of extracellular CA in
vitro and in vivo (10, 11, 17). This report represents an
investigation of the presence and function of branchial
membrane-associated CA in the blue crab, Callinectes
sapidus, utilizing QAS in the intact organism.

MATERIALS AND METHODS

Collection and maintenance of animals. Adult male blue
crabs, Callinectes sapidus, were obtained from commer-
cial watermen in Ocean Springs, MI. Animals were main-
tained in large (100 gal) Plexiglas aquaria equipped with
biological filters. Salinity was maintained at ~12 ppt
(350 mosmol). Blue crabs were fed daily on chicken liver
but were starved for 48 h prior to being used in an
experiment.

Hemolymph acid-base measurements. Individuals were
placed in 2-l Plexiglas flow-through chambers. Aerated
and filtered water was circulated through the chamber
from a 35-gal reservoir. The chambers were covered with
black plastic to minimize disturbances to the animals.
After 24 h in the chamber a prebranchial (venous) blood
sample was withdrawn anaerobically from the infrabranch-
ial sinus. Hemolymph pH and PCO2 were measured on
a Radiometer pHM/73 blood gas analyzer (G297 glass
capillary electrode and K497 reference, and E5036-0 CO2
electrode). Total CO2 (CT) was measured on 20 µl of
hemolymph using a Capni-Con 3 total CO2 analyzer
(Cameron Instruments). Following the initial measure-
ments individuals were either injected with crab Ringer
saline (controls) or with QAS (1- or 10-mM concentra-
tion in the hemolymph), an inhibitor that is known not
to permeate the gill membrane (10). Hemolymph samples
were taken at various times after the injection, and pH,
PCO2, and CT were measured.

This procedure was repeated using low concentrations
(10 µM) of two other CA inhibitors: acetazolamide and
benzolamide.

Hemolymph osmotic and ionic measurements. A second
set of animals was treated in the same manner as de-
scribed above. These crabs were given injections of 1 mM
QAS or 10 mM QAS, and hemolymph osmotic and ionic
concentrations were measured on samples taken over 96
h postinjection. Osmolality was determined on a vapor
pressure osmometer (Wescor 5100C). Hemolymph chlo-
ride (Cl-) was measured coulometrically (Buchler-Cot-
love Chloridometer); Na+ and K+ were determined by
flame photometry (Radiometer FLM 3). All experiments
were performed at 23 ± 1°C. These values were then
compared with those obtained from blue crabs treated
with 1 mM acetazolamide, a CA inhibitor known to
permeate the gill membrane (15).

RESULTS

Effect of QAS on blue crab hemolymph osmotic and
ionic concentrations. QAS, at an initial concentration of
1 or 10 mM in the hemolymph of the blue crab, had no
effect on either the total hemolymph osmolality or on
the concentrations of the two major ions, Na+ and Cl-
(Fig. 1). Throughout the 96-h time course after the QAS
injection hemolymph osmolality values were not signifi-
cantly different from preinjection values or from those
of the saline-injected controls (P > 0.05, F test). Initial
concentrations of QAS were maintained for only ~4 h
after injection; the compound was then slowly cleared
from the hemolymph over the following 96 h (10, 11).
For an initial concentration of 10 mM QAS, however,
hemolymph values remained >2 mM through 48 h, and
no perturbation of hemolymph osmolality occurred (Fig.
1).

An injection of acetazolamide (1 mM), on the other
hand, resulted in a significant reduction in hemolymph
osmolality by 2-4 h postinjection; osmolality then
remained depressed through 48 h (Fig. 1).

FIG. 1. Effects of 1 mM quaternary ammonium sulfanilamide (QAS) (filled circles, solid line), 10 mM QAS (filled squares, dashed lines), and
1 mM acetazolamide (open squares, broken line) on prebranchial hem-
olymph osmolality, Na, and Cl concentrations in C. sapidus. Open
circles represent crab Ringer saline-injected controls; temperature was
22-23°C. Values are means ± SE; n = 6. Acetazolamide data redrawn
from Henry and Cameron (15).
The same pattern was seen for hemolymph Na⁺ and Cl⁻ concentrations. Neither 1 nor 10 mM QAS had any effect on hemolymph Na⁺ and Cl⁻ concentrations at any time, whereas the QAS was present in the hemolymph (Fig. 1). Treatment with acetazolamide (1 mM) caused the concentrations of both ions to drop.

**Effect of QAS on blue crab hemolymph acid-base parameters.** QAS (1 or 10 mM) produced a near-immediate respiratory acidosis in the hemolymph of the blue crab (Fig. 2). Prebranchial hemolymph pH was lowered by ~0.25 units during the initial 30 min after the injection of the inhibitor; PCO₂ rose by ~2 Torr, and CT also was elevated by ~2 mM. The acidosis was apparent at 10 min postinjection and reached its maximum value at 30 min. For 1 mM QAS hemolymph acid-basis status began to return to normal after 2 h, and the acidosis was completely over by 24 h (Fig. 2). The higher concentration of QAS produced an acidosis of approximately equal magnitude but of longer duration; for 10 mM QAS, hemolymph acid-base balance was not restored until 72 h after treatment.

The results obtained for QAS were then compared with those for two other CA inhibitors: acetazolamide, which is known to permeate biological membranes, and benzolamide, which is believed to cross membranes much more slowly because of its low lipid solubility (21). Because these two inhibitors have a higher affinity for the enzyme than does QAS, lower concentrations (10 μM) were used. The effects were basically the same as those for QAS; a respiratory acidosis developed early after injection of the inhibitor (Fig. 3). The acidosis was again of approximately equal magnitude to that caused by QAS. The duration, however, was much shorter, especially for acetazolamide. By 4 h after treatment with acetazolamide hemolymph acid-base parameters were already returning to preinjection, control values with PCO₂ values showing no significant difference from controls (P > 0.05, Student’s t test). The effects of benzolamide were almost parallel to those of acetazolamide (Fig. 3).

**DISCUSSION**

The effects of QAS on the hemolymph osmotic and ionic concentrations and on acid-base status show first, that CA is present on the basal membrane of the gill and that the enzyme is exposed to the hemolymph. The physiological significance of the membrane-associated CA appears to be in facilitating CO₂ excretion across the gills by mobilizing hemolymph HCO₃⁻ to CO₂, maintaining an adequate PCO₂ gradient across the gill lamellae. When this enzyme is selectively inhibited by QAS a respiratory acidosis quickly develops. Branchial CO₂ excretion is initially retarded, and CO₂ accumulates in the hemolymph until a new equilibrium is established at a higher PCO₂; the increase in hemolymph PCO₂ ensures that normal CO₂ excretion will continue. By use of an isolated perfused gill preparation from Cancer productus, Burnett and McMahon (3) have shown that perfusion with DBI reduces the amount of 14C-labeled HCO₃⁻ that is mobilized from the perfusion medium across the gill, independently proving the existence of branchial membrane-associated CA and describing its function. Burnett et al. (4) reported that no extracellular branchial CA could be detected using an electrometric CA assay of large gill fragments. Extracellular membrane-associated CA in other tissues comprises only a small fraction of the total CA activity of the tissue (5–10%) (16, 30); thus it is possible that the amount of branchial membrane-associated CA was below the level of sensitivity of the assay. It is also possible that, since relatively large pieces of gill tissue were used, the assay was limited by the diffusion of CO₂ and H⁺ and not by the catalyzed reaction.

In this report the observed respiratory acidosis is neither severe nor prolonged. It was, however, of approxi-
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FIG. 3. Effects of 10 μM acetazolamide (closed circles, solid line) and 10 μM benzolamide (filled squares, broken line) on prebranchial hemolymph Pco₂, pH, and total CO₂ (Ct) in C. sapidus. Open circles represent crab Ringer saline-injected controls; temperature was 22–23°C. Values are means ± SE; n = 4–6.

Morphologically the same magnitude and duration as that reported for another crustacean, Cancer productus (23). Also there was no dose-dependent response for the two concentrations of QAS used (1 and 10 mM); this would suggest

that 1 mM QAS is more than sufficient to inhibit the total complement of extracellular branchial membrane-associated CA. The comparative effects of low (10 μM) concentrations of acetazolamide and benzolamide support this idea as both inhibitors caused a respiratory acidosis of virtually identical magnitude to that produced by QAS. It is possible that a relatively mild respiratory acidosis is the result of the inhibition of only a small percentage of the total gill CA activity that constitutes the membrane-associated fraction. In other respiratory and ion-transporting tissues (e.g., vertebrate lung and kidney) membrane-associated CA activity makes up only 5–10% of the total CA activity of that tissue (16, 22, 30).

Since crustaceans have no hemolymph CA (12, 13), they would depend entirely on extracellular membrane-associated branchial CA for facilitating CO₂ excretion. Aquatic vertebrates possess both branchial and erythrocyte CA, and when both are inhibited by methazolamide, the resultant respiratory acidosis is much more severe (26).

The comparatively mild nature of the inhibitor-induced acidosis in the blue crab might be explained by other factors as well. For animals at rest, the respiratory role of branchial CA might be minimal. Mobilization of hemolymph HCO₃⁻ by the membrane-associated enzyme fraction may become more important during exercise when CO₂ buildup in the hemolymph is greater and the rate of CO₂ excretion is also higher. Another possibility is that the sulfonamide compounds might influence the ventilation-perfusion parameters in the blue crab and that a component of the acidosis may be unrelated to branchial CA inhibition. Existing evidence, however, does not support the latter suggestion, as acetazolamide

FIG. 4. Model of compartmentalized carbonic anhydrase (CA) function in blue crab gill showing both membrane-associated CA and cytoplasmic CA. Dashed lines represent diffusion; solid lines represent some form of coupled transport. Large arrows indicate predominant direction of CA-catalyzed reactions.
had no effect on ventilation, heart rate, or hemolymph oxygenation in another species of crustacean (3). Regardless both possibilities deserve further investigation.

The fact that QAS, acetazolamide, and bezolamide all produce a rapid but relatively short-lived respiratory acidosis can explain some of the past confusion concerning the role of branchial CA. Previous studies involved the use of high dosages of acetazolamide (1 mM) designed to inhibit the total complement of branchial CA. Burnett et al. (4) waited 12 h after treatment before measuring hemolymph pH and reported an alkalosis; they did not measure PCO₂. Henry and Cameron (15) took their initial PCO₂ measurement at 6 h after an acetazolamide injection. Thus, in both cases, the initial respiratory acidosis was missed. Also by 6 h after administration of high doses of acetazolamide, the inhibitor has permeated the gill to the point where it completely inhibits cytoplasmic CA (15), disrupting the branchial ion regulatory mechanism and causing a hemolymph alkalosis to occur as a result of a shift in the strong-ion difference toward the positive direction (15). When this effect is at its maximum, 24 h postinjection (16), acetazolamide in the hemolymph is virtually undetectable (10), and presumably the extracellular membrane-associated CA is no longer inhibited. Thus it appears that a permeable inhibitor such as acetazolamide has a biphasic effect on the gill; initially, when the inhibitor concentration is high in the hemolymph and low or absent in the intracellular compartment, membrane-associated CA is inhibited, HCO₃⁻ mobilization from hemolymph is impeded, and a respiratory acidosis results. Over time, as the inhibitor is cleared from the hemolymph and enters the branchial tissue, cytoplasmic CA is inhibited, resulting in the development of a nonrespiratory alkalosis at the same time that membrane-associated CA function is being restored. A membrane-impermeant CA inhibitor, such as QAS, only affects the respiratory function of branchial CA; QAS has no effect on hemolymph osmotic and ionic concentrations. At 24 h following an injection of 1 and 10 mM QAS, hemolymph concentrations of the inhibitor are ~600 µM and 4 mM, respectively, cytoplasmic CA is uninhibited (10), and hemolymph ionic parameters are undisturbed (Fig. 1). In contrast, at 24 h after an injection of 1 mM acetazolamide, hemolymph concentration of the drug is virtually zero (10), branchial CA is completely inhibited, and hemolymph osmotic and ionic concentrations are significantly lowered (15). These results confirm the role of intracellular branchial CA in the ion regulatory process and also provide a convenient bioassay for testing the permeability of the branchial membrane to different CA inhibitors in vivo, at least in ion-regulating species. If the inhibitor causes a disruption in hemolymph ion concentrations, it must be crossing the basal branchial membrane and acting on the intracellular CA.

Thus what emerges is a picture of branchial CA that is compartmentalized within different subcellular fractions and has different physiological roles (Fig. 4). The membrane-associated CA plays a respiratory role, mobilizing HCO₃⁻ to CO₂, whereas the cytoplasmic CA, through the hydration of CO₂ to H⁺ and HCO₃⁻, is important in the branchial ion-transport mechanisms. The distribution of branchial CA allows for a very efficient cycle of extracellular dehydration and intracellular hydration and is a good example of how an enzyme can be involved in multiple physiological processes in a single tissue. The basal membrane of fish gills is known to be impermeable to HCO₃⁻ (24) and, given their anatomical and physiological similarities, it is reasonable to assume that the basal membrane of the crustacean gill is also HCO₃⁻ impermeable. CA facilitates the transport of HCO₃⁻ across membranes only when it is on the "upstream" side where it maintains a high PCO₂ in the unstirred layer through the catalyzed dehydration reaction (9). Thus, for branchial CA to facilitate CO₂ excretion, it must have direct access to hemolymph HCO₃⁻, a condition that is satisfied by extracellular membrane-associated CA. Mass action (relatively high hemolymph HCO₃⁻ and the removal of the product, CO₂, by diffusion) would ensure that the dehydration reaction predominates in the hemolymph. In the cytoplasm the reverse would occur; H⁺ and HCO₃⁻ are continuously being transported out of the cell while CO₂ is diffusing in, driving the reaction in the direction of hydration.

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