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FACILITATION OF CO_2 EXCRETION BY CARBONIC ANHYDRASE LOCATED ON THE SURFACE OF THE BASAL MEMBRANE OF CRAB GILL EPITHELIUM

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Abstract. An isolated perfused crab gill preparation was used to test the hypotheses that crab gill carbonic anhydrase (CA) catalyzes the efflux of CO_2 from the hemolymph, which lacks the enzyme, to the ambient medium and that the CA is localized on the luminal surface of the basal membrane. It was found that the efflux of CO_2 from the internal perfusate was sensitive to the flow rate of the internal perfusate through the gill (and thus the residence time within the gill). The sensitivity of the CO_2 efflux to residence time was nearly abolished upon treatment of the gill with an impermeable dextran-bound CA inhibitor. It is concluded that CA present on the luminal surface of the gill epithelium facilitates CO_2 excretion by catalyzing the dehydration of the large hemolymph bicarbonate pool to the more diffusible molecular CO_2. The action of the enzyme is important in maintaining a CO_2 gradient between hemolymph and water in a situation where hemolymph P_{CO_2} is normally low, water P_{CO_2} is variable, and the gills themselves are a source of metabolic CO_2.

Acetazolamide   Carbonic anhydrase   Dextran-bound inhibitor
Bicarbonate CO_2 excretion   Gill

Like the lungs and gills of other animals, the gills of decapod crustaceans possess carbonic anhydrase (CA) (Henry, 1984). However, unlike vertebrate air and water breathers, the hemolymph of crustaceans has no CA activity and therefore provides a useful model for studying the general problem of respiratory elimination of CO_2. The epithelium of the crab gill is one cell thick and bound on the outside apical membrane by chitin. Recently, it has been proposed (Burnett, 1984; Burnett *et al.*, 1985) that CA occurs at two distinct locations within the gill. One proposed location is intracellular and the other, the cell surface of the basal membrane. CA at each location is assumed to be associated with a different function. Intracellular CA would be involved primarily with the hydration of CO_2 to HCO_3^- and H^+, which may serve as counterions for the

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inward pumping of Na^+ and Cl^- (Henry and Cameron, 1983). Cell surface CA would be located on the luminal surface of the basal membrane, where the enzyme has access to the large hemolymph bicarbonate pool and is involved primarily in the dehydration of HCO_3^- to the more diffusible CO_2 for respiratory elimination (Burnett 1984; Burnett *et al.*, 1985). However, the hypothesis that CA is present on the hemolymph side of the gill has not previously been tested directly.

In this experiment, we test the hypotheses that CA is bound to the luminal surface of the basal membrane of crab gill epithelial cells and that CA in this location facilitates the efflux of CO_2 by catalyzing the dehydration of hemolymph bicarbonate. These hypotheses are tested by measuring the efflux of ^{14}C (originating as HCO_3^- and CO_2) from the hemolymph of an isolated perfused gill as a function of hemolymph residence time within the gill and before and after treatment with a dextran-bound CA inhibitor (DBI) which selectively inhibits only CA bound to the cell surface. If CA acts to facilitate CO_2 excretion by dehydrating the large hemolymph bicarbonate pool, then CO_2 efflux from the internal perfusate must be sensitive to the residence time within the gill and relatively insensitive to residence time after treatment with a CA inhibitor. In the presence of a CA inhibitor the dehydration reactions occur at the slow uncatalyzed rate, which Aldridge and Cameron (1979) suggest is the rate-limiting step in the process of CO_2 elimination across crab gills. Finally, if the flux of CO_2 from the internal perfusate is sensitive to a CA inhibitor that is impermeable to the basal membrane, then CA is localized on the surface of the cell.

Materials and methods

Red rock crabs *Cancer productus* (males; mean weight = 439 g, range 271–552 g) were collected at the Bamfield Marine Station, British Columbia, and transported to Calgary where they were held in well aerated sea water at 12 °C. Crabs were fed smelt 3 times each week.

In vitro inhibition of CA by the dextran-bound inhibitor. A dextran-bound inhibitor (DBI) of CA (MW = 5000–6000 and synthesized from the glutaryl analog of 2-succinylamido-1,3,4-thiadiazole-5-sulfonamide (Tinker *et al.*, 1981) was added to crude homogenates of crab gills. The homogenates were then assayed for CA activity using the method of Burnett *et al.* (1981). The method consists of following the rate of appearance of protons when saturating amounts of CO_2 are added to tissue homogenates. A small volume of homogenate was added to 15 ml of 50 mM HEPES (Sigma) and the rate of decline from pH 8.1 to 8.0 was followed when 10 ml of CO_2-saturated water was rapidly injected into the mixture. Assays were carried out at 12 °C.

Gill permeability to DBI. The permeability of the gill to DBI was tested directly. We were unable to demonstrate that DBI passed across the epithelium. In experiments where we perfused gills with a high concentration of DBI (2.5 mg/ml), we could not

detect its presence in a small volume (8 ml) of rapidly stirred sea water in which the perfused gill was incubated for a period of up to 2 h. The presence of DBI in the sea water was determined by adding the sea water to solutions of CA (Sigma) and measuring the activity of the enzyme solution. If DBI was present in the sea water, CA activity was reduced. In this manner we were able to detect the presence of as little as 0.0025 mg DBI/ml sea water.

Measurement of CO_2 fluxes. Gill 7 (Pearson, 1908) was excised and perfused using techniques described by Burnett (1984). The composition of the internal perfusate has been previously described (Burnett, 1984) and included 1% crab hemolymph. The P_{O_2} of the internal perfusate was adjusted to 10 Torr and the P_{CO_2} to 3.4 Torr using gases delivered by Wösthoff gas mixing pumps. Radiolabeled CO_2 was introduced to the internal perfusate as $NaH^{14}CO_3$ at least 30 min prior to the beginning of an experiment.

The efflux of ^{14}C (originating as both HCO_3^- and CO_2 in equilibrium at pH 7.9, P_{CO_2} 3.4 Torr, and 12 °C) from the internal perfusate was measured as a function of the flow rate of the internal perfusate. Samples (1 ml) of the internal perfusate were first collected immediately after passing through the gill. Similar samples were then collected just prior to passing through the gill. The backflux of ^{14}C from the medium to the internal perfusate was considered negligible, since the flow rate of the sea water past the gill was many times faster than the flow of internal perfusate through the gill (*cf.* sea water flow = 27 ml/min and internal perfusate flow = 1–3 ml/min). Thus, ^{14}C fluxing across the gill was rapidly diluted in a large volume of sea water. Neither the internal perfusate nor the sea water ventilating the gill were recirculated. The entire 1 ml sample was injected into the outer well of a 50 ml flask which contained a center well with 0.3 ml Protosol (New England Nuclear) to absorb CO_2. The flask was immediate stoppered and all forms of CO_2 in the perfusate were volatilized by injecting 0.1 ml of 70% perchloric acid through a thick rubber diaphragm into the outer well. The flask was gently shaken for at least 1 h after which time the Protosol (now containing ^{14}C activity) was removed, placed in 3 ml of scintillation fluid (Econofluor, New England Nuclear) and counted using a liquid scintillation counter. We had previously determined that 1 h was adequate time for maximum absorption of ^{14}C activity into the Protosol.

CO_2 fluxes were measured at three different internal perfusate flow rates to determine the sensitivity of the CO_2 efflux to the residence time of perfusate within the gill (Burnett *et al.*, 1985). Next, DBI was freshly dissolved and added to the internal perfusate. DBI, because of its size, has a very low membrane permeability and has been used previously in perfusion experiments to selectively inhibit CA bound to the brush border of the proximal tubules of rat kidneys (Lucci *et al.*, 1983). Gills were perfused with the DBI-perfusate for 20–30 min and CO_2 fluxes were again measured at the same three flow rates. In all cases, the gills were exposed to DBI for no more than 2 h. Henry and Cameron (1983) have shown that CA activity in gill homogenates was inhibited 100% by acetazolamide only after a 4 h incubation period in the blue crab, *Callinectes sapidus*, a result they attributed to the slow permeation of the inhibitor across the basal membrane. While we have not demonstrated that DBI is impermeable to the gill

epithelium, the much larger size of DBI (MW = 5000–6000) compared to acetazolamide (MW = 244) would make its permeation much slower. Therefore, we conclude that the DBI during the treatment period used in this experiment had access only to cell surface CA.

Results and Discussion

CO_2 residing in the hemolymph of crab gills fluxes across the gills into the ambient medium, but the flux depends primarily on the rate of dehydration of the non-permeable bicarbonate ions to the permeable molecular CO_2 in the hemolymph (Aldridge and Cameron, 1979). According to a computer simulation developed by Aldridge and Cameron (1979), there will be an initial large efflux of CO_2 from the hemolymph as it enters the gill, its source the pool of molecular CO_2 already present. It is after this initial flux, which the model predicts lasts about 1 sec, that the CO_2 movement across the gill becomes dependent upon the rate of dehydration of the hemolymph bicarbonate pool. Using the Aldridge and Cameron model and adjusting the uncatalyzed rate constants to account for the lower temperatures used in this study, we predict that only 2.8% and 6.5% of the total CO_2 present in the hemolymph fluxes across the gill into the ambient medium after residing in the gill for 15 and 90 sec, respectively. Thus, the efflux of CO_2 from the hemolymph should be relatively insensitive to the time it resides within the gill. On the other hand, if CA present in the gill has access to hemolymph bicarbonate, then CO_2 efflux should show a greater sensitivity to residence time than found in the uncatalyzed situation (Burnett *et al.*, 1985).

The experimental procedure used in this study is designed specifically to test two hypotheses: (1) that CO_2 efflux in the untreated gill is sensitive to the hemolymph residence time within the gill, and (2) that the CO_2 efflux is CA mediated with the CA being located on the luminal surface of the basal membrane of the gill epithelium.

The specific probe for cell surface CA was a dextran-bound CA inhibitor (DBI). DBI is a potent inhibitor of CA activity in gill homogenates with an I_{50} of 0.165 μg/ml (fig. 1). In the gill perfusion experiments we routinely added 1.25 mg/ml DBI to the internal perfusate. However, in one perfusion experiment a partial inhibition of the CO_2 flux was observed at the lowest perfusion flow rate (longest residence time in the gill) when

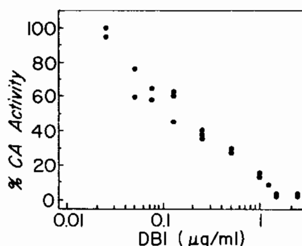

Fig. 1. Dose–response curve for CA activity in the presence of the dextran-bound inhibitor (DBI). CA activity was determined using the method described by Burnett *et al.* (1981).

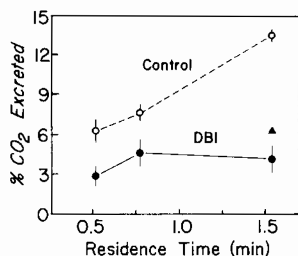

Fig. 2. Percent CO_2 excreted as a function of the residence time of the internal perfusate (containing the ^{14}C label) within the gill. CO_2 excreted represents a unidirectional efflux from the internal perfusate. All data are reported as mean $\pm$ SE (N = 5). DBI concentration is 1.25 mg/ml, except for a single observation (▲) where DBI concentration is 0.395 mg/ml. Residence time was determined by estimating the gill hemolymph volume based on surface area estimates of gills of similar size (after Aldridge and Cameron, 1979).

0.395 mg/ml DBI was present (fig. 2), suggesting that the efflux of label was dose dependent.

In confirmation of previous results (Burnett *et al.*, 1985), the efflux of CO_2 from the perfusate was sensitive to the residence time of the perfusate within the untreated gill (fig. 2). When the gill was treated with DBI, the CO_2 efflux was significantly reduced at all residence times ($P < 0.01$ according to a one-tailed *t*-test). A comparison of the CO_2 efflux with oxygen uptake into the internal perfusate before and after treatment with acetazolamide in a previous study indicated that the large decreases in CO_2 flux are due to a specific effect of CA inhibition and not an effect of the drug on the degree or pattern of gill perfusion (Burnett *et al.*, 1985). Since DBI has access only to CA present on the external surface of the basal membrane of the gill, we conclude that the active site of the enzyme faces the lumen of the gill hemolymph space. These results are quantitatively similar to those obtained in another *Cancer* species using the CA inhibitor acetazolamide (Burnett *et al.*, 1985). However, acetazolamide has been shown to be relatively permeable to cell membranes (Holder and Hayes, 1965) and the possibility that inhibiting intracellular CA could account for the results of their experiment cannot be eliminated.

Information obtained from other studies of crab gills supports the concept of two separate pools of CA, each serving different functions. Acetazolamide has been used exclusively in studies of crab gill CA function and its penetration into epithelial cells is slow (Henry and Cameron, 1983). Henry and Cameron (1983) injected blue crabs, *C. sapidus*, with acetazolamide, then removed the gills after varying periods of time and assayed gill homogenates for CA activity. Their results showed that the longer the incubation period in the live animal, the greater the reduction of CA activity in the homogenates. They attributed this result to a slow permeation of acetazolamide across the basal membrane of the gill epithelium. These results suggest that a fairly large pool of CA is intracellular and not immediately accessed by the inhibitor. In a different study, Burnett (1984) demonstrated that the efflux of CO_2 from the internal perfusate flowing through an isolated perfused gill was reduced by half 15 min after treating the gill with

acetazolamide. Given the slow penetration of acetazolamide into the gill epithelium (Henry and Cameron, 1983), the rapid reduction in CO_2 flux must be attributed to CA present on the cell surface or near the basal membrane and functioning in CO_2 excretion (Burnett, 1984).

The presence of CA on the luminal surface of the crab's respiratory epithelium is interesting in that this location parallels that found on the capillary endothelium of mammalian lungs (see Swenson, 1984, for review). Moreover, CA is thought to occur cytoplasmically in both groups. Different mechanisms have been suggested to account for the action of CA in facilitating CO_2 fluxes across epithelia involving cytoplasmic and membrane-associated enzymes. In the present study, the mechanism of rapid interconversion between CO_2 and HCO_3^- within the gill epithelium by the cytoplasmic enzyme as a mode of facilitation (Longmuir *et al.*, 1966; Enns, 1967; Ward and Robb, 1967) can be eliminated, since DBI selectively inhibits only CA on the basal membrane. The facilitation process in crab gills must therefore be due largely to the basal membrane CA. Given the natural lack of CA in the hemolymph, and that the uncatalyzed slow dehydration of hemolymph bicarbonate limits CO_2 excretion in crabs (Aldridge and Cameron, 1979), the mechanism of facilitation is the catalysis of the dehydration of hemolymph HCO_3^- to the more diffusible CO_2 by CA located on the luminal surface of the basal membrane. A similar strategy has also been proposed for pulmonary systems (Klocke, 1978).

Normally, hemolymph resides in gills for 3–15 sec (Aldridge and Cameron, 1979; and based on our own measurements of gill hemolymph volume after Aldridge and Cameron (1979) and cardiac output). The residence times used in this experiment are probably rarely encountered in nature, but serve to amplify the action of the cell surface CA. There is abundant evidence that the species used in this experiment (McMahon *et al.*, 1984) and other crab species (Burnett *et al.*, 1985) normally rely on gill CA for CO_2 elimination. In the CO_2 excretion process, the driving force for CO_2 movement across gills is the molecular CO_2 concentration difference between hemolymph within the gills and water bathing the gills. Cell surface CA serves to maximize this concentration difference which is smaller than previously assumed (Aldridge and Cameron, 1979). Two important factors serve to lower the driving force for the net flux of CO_2. First, gills are metabolically active tissues, producing CO_2 endogenously (Pressley *et al.*, 1981) and creating a barrier to CO_2 diffusion from the hemolymph to the water. Furthermore, gills perfused with ^{14}C-labeled lactate or glucose show unidirectional fluxes of CO_2 into both the hemolymph and the sea water bathing the gill (Burnett and Grieshaber, unpublished data). Second, partial pressures of CO_2 in the ambient medium are variable and can be close to that found in crab hemolymph (*e.g.* 10^{-4} to 3 Torr; Truchot and Duhamel-Jouve, 1980). Since the partial pressures of CO_2 in crab hemolymph are normally low (2–4 Torr), CA catalyzed recruitment of molecular CO_2 from the large hemolymph bicarbonate pool is necessary to maintain a favorable gradient between the hemolymph and water for the net removal of CO_2.

The occurrence of CA in the respiratory tissues of two such evolutionarily different groups as crustaceans and vertebrates is of particular interest when it is recognized that

crabs have no circulating CA while vertebrates have an abundance of the enzyme in the red blood cells. Gill CA is clearly important to the crabs in the excretion of CO_2 and the regulation of hemolymph ion concentrations. While the latter function does not occur in mammalian lungs, CA is of potential importance in facilitating CO_2 elimination in both groups, but it may be of greater importance in the crab and in all other respiratory systems which contain no circulating carbonic anhydrase.

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