FACILITATION OF CO₂ 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 hypothesis that crab gill carbonic anhydrase (CA) catalyzes the efflux of CO₂ 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 CO₂ 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 CO₂ efflux to residence time was nearly abolished upon treatment of the gill with an irreversible dextrose-bound CA inhibitor. It is concluded that CA is present on the luminal surface of the gill epithelium facilitates CO₂ excretion by catalyzing the dehydration of the large hemolymph bicarbonate pool to the more diffusable molecular CO₂. The action of the CA is important in maintaining a CO₂ gradient between hemolymph and water in a situation where hemolymph P₃O₂ is normally low, water P₃O₂ is variable, and the gills themselves are a source of metabolic CO₂.

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 CO₂. 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 CO₂ to HCO₃⁻ and H⁺, which may serve as counterions for the
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 HCO₃⁻ to the more diffusible CO₂ 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 CO₂ by catalyzing the dehydration of hemolymph bicarbonate. These hypotheses are tested by measuring the efflux of 14C (originating as HCO₃⁻ and CO₂) 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 CO₂ excretion by dehydrating the large hemolymph bicarbonate pool, then CO₂ efflux from the internal perfusate must be sensitive to the residence time within the gill and relatively insensitive to residence time before 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 CO₂ elimination across crab gills. Finally, if the flux of CO₂ 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 Bumfield Marine Station, British Columbia, and transported to Calgary where they were held in well aerated sea water at 12 ºC. 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 CO₂ 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 CO₂-saturated water was rapidly injected into the mixture. Assays were carried out at 12 ºC.

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 reduced. In this manner we were able to detect the presence of as little as 0.0025 ng DBI/ml sea water.

**Measurement of CO₂ 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 15% crab hemolymph. The PO₂ of the internal perfusate was adjusted to 10 Torr and the PCO₂, to 3.4 Torr using gases delivered by Worthington gas mixing pumps. Radiolabeled CO₂ was introduced to the internal perfusate as NaH²¹⁴CO₃ at least 30 min prior to the beginning of an experiment. The efflux of ¹⁴C (originating as both HCO₃⁻ and CO₃²⁻, in equilibrium at pH 7.9, PO₂, 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 ¹⁴C 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, ¹⁴C fluxing across the gill was rapidly diluted in a large volume of sea water. Neither the internal perfusate nor the sea water sampling 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 CO₂. The flask was immediately stoppered and all forms of CO₂ 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 the Protosol (now containing ¹⁴CO₂ activity) was dissolved, 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 ¹⁴C activity into the Protosol.

CO₂ fluxes were measured at three different internal perfusate flow rates to determine the sensitivity of the CO₂ 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 (Lucchi et al., 1983). Gills were perfused with the DBI-perfusate for 20–30 min and CO₂ 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...
CO₂ residing in the hemolymph of crab gills flows 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 CO₂ 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 CO₂ from the hemolymph as it enters the gill, its source: the pool of molecular CO₂ already present. It is after this initial flux, which the model predicts lasts about 1 sec, that the CO₂ 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% of the total CO₂ present is 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 CO₂ from the hemolymph should be relatively insensitive to the time it resides within the gill.

On the other hand, if CA is present in the gill, access to hemolymph bicarbonate, then CO₂ efflux should show a greater sensitivity to residence time than found in the uncatalyzed system (Burnett et al., 1985).

The experimental procedure used in this study is designed specifically to test two hypotheses: (1) that CO₂ efflux in the untreated gill is sensitive to the hemolymph 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 IC₅₀ 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 CO₂ 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).]
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 CO₂ 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 CO₂ efflux was significantly reduced at all residence times (P < 0.01 according to a one-tailed t-test). A comparison of the CO₂ efflux with oxygen uptake into the internal perfusate before and after treatment with azacizolamide in a previous study indicated that the large decreases in CO₂ flux are due to a specific effect of CA inhibition and not an effect of the drug on the pattern or degree 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 observed in another Cancer species using the CA inhibitor azacizolamide (Burnett et al., 1985). However, azacizolamide 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. Azacizolamide has been used exclusively in studies of crab gill CA function and its penetration into epithelial cells is slow (Henry and Cameron, 1981). Henry and Cameron (1983) injected blue crabs, C. sapidus, with azacizolamide, 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 azacizolamide across the basal membrane of the gill epithelium. These results suggest that a fairly large pool of CA is intracellular and not immediately accessible by the inhibitor. In a different study, Burnett (1984) demonstrated that the efflux of CO₂ 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 CO₂ flux must be attributed to CA present on the cell surface, or near the basolateral membrane and functioning in CO₂ 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 CO₂ flux across epithelia involving cytoplasmic and membrane-associated enzymes. In the present study, the mechanism of rapid interconversion between CO₂ and HCO₃⁻ within the gill epithelium by the cytoplasmic enzyme as a mode of facilitation (Longmuir et al., 1966; Entis, 1967; Ward and Robb, 1967) can be eliminated, since DBI selectively inhibits only CA on the basolateral membrane. The facilitation process in crab gills must therefore be due largely to the basolateral membrane CA. Given the natural lack of CA in the hemolymph, and that the uncontrolled slow desorption of hemoltaphic bicarbonate limits CO₂ excretion in crabs (Aldridge and Cameron, 1979), the mechanism of facilitation is the catalysis of the dehydration of hemolymph HCO₃⁻ to the more diffusible CO₂ by CA located on the luminal surface of the basolateral membrane. A similar strategy has also been proposed for pulmonary systems (Knueke, 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 CO₂ elimination. In the CO₂ excretion process, the driving force for CO₂ movement across gills is the molal CO₂ 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 facets serve to lower the driving force for the net flux of CO₂. First, gills are metabolically active tissues, producing CO₂ endogenously (Pressler et al., 1981) and creating a barrier to CO₂ diffusion from the hemolymph to the water. Furthermore, gills perfused with ¹⁴C-labeled lactate or glucose show unidirectional fluxes of CO₂ into both the hemolymph and the water bathing the gills (Burnett and Grieshaber, unpublished data). Second, partial pressures of CO₂ in the ambient medium are variable and can be close to that found in crab hemolymph (e.g. 10⁻¹ to 3 Torr; Trutner and Doihamele-Nay, 1980). Since the partial pressures of CO₂ in crab hemolymph are normally low (2–4 Torr), CA catalyzed recruitment of molecular CO₂ from the large hemolymph bicarbonate pool is necessary to maintain a favorable gradient between the hemolymph and water for the net transfer of CO₂.

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 CO₂ and the regulation of hemolymph ion concentrations. While the latter function does not occur in mammalian lungs, CA is of potential importance in facilitating CO₂ 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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References


