The Function of Carbonic Anhydrase in Crustacean Gills

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1 Introduction

Carbonic anhydrase (CA) is one of the most ubiquitous enzymes found in living organisms. The major action of the enzyme is the catalysis of the reactions of carbon dioxide with water, reactions which proceed uncatalyzed at very slow rates even at physiological temperatures. The enzyme is important for several reasons. It can mobilize rapidly carbon dioxide to bicarbonate and carbonate, which has implications for acid-base balance, ion transport, and calcification, all at the tissue and cellular levels. It can also mobilize bicarbonate to the more diffusible carbon dioxide, facilitating transport within, between, and across tissues and cells. Among animals CA has been studied extensively in the vertebrates and especially mammals. Although the occurrence of CA among the invertebrate phyla has been known for some 47 years (Ferguson et al. 1937), it is only recently that investigations into its function in invertebrates have begun in earnest. The reader is referred to a recent review of the subject by Henry (1984). The purpose of this article is to present information on what is currently known about the distribution of CA and its function in transporting substances across gills in a single group of invertebrate organisms, the decapod crustaceans.

2 Distribution

Carbonic anhydrase is found in its highest concentration in the gill tissues, but is also present in muscle, heart, epithelial lining of the branchial chamber, and green gland (Randall and Wood 1981; Henry and Cameron 1982b; McMahon et al. 1984). The complete absence of CA from circulating hemolymph has interesting implications for CO_2 transport which will be discussed below. There is a strong correlation between the activity of CA and the ion pumping function of specific gills in species which regulate hemolymph ionic concentration in dilute media. When the blue crab *Callinectes sapidus* is transferred from seawater to a dilute medium, the onset of an increase in CA activity occurs simultaneously with increases in both Na^+/K^+ ATPase activity (Towle et al. 1976; Neufeld et al. 1980) and bicarbonate ATPase activity (Lee 1982). Thus, the enzyme's distribution leaves little doubt as to a function in ionic regulation. In addition, the

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presence of CA in the gills not associated with ion pumping in osmoregulating crustaceans and in the gills of stenohaline, osmoconforming species is consistent with a CO_2 excretion function as discussed below.

3 Ionic Regulation

The role of the crustacean gill in regulating hemolymph sodium and chloride ion concentrations has been well established and is discussed elsewhere in this symposium (see contribution by Gilles and Péqueux in the present volume). Kirschner (1983) has reviewed recently the mechanisms of ion transport in different types of epithelia in various groups of animals. The overall picture of ion transporting mechanisms in crustacean gills does not appear to be different from that found in other groups of animals where ions are transported from the ambient medium to the blood or hemolymph (Fig. 1). Sodium ions are pumped across the basolateral membrane (adjacent to the hemolymph) from the interior of the cell into the hemolymph in exchange for potassium ions. This process is associated with Na^+/K^+ ATPase activity which is stimulated in posterior gills when euryhaline crabs are transferred to dilute media (Towle et al. 1976; Neufeld et al. 1980; Péqueux et al. 1984). Amiloride sensitive sodium channels occur along the apical surface (adjacent to the external medium) and allow sodium ions to pass into the cell from the medium (Cameron 1979a; Pressley et al. 1981). This step in the process is thought to be electrically neutral with H^+ and/or NH_4^+ serving as counterions.

Less is known of the chloride uptake mechanism, but as in other epithelia, chloride uptake occurs independently of sodium uptake (Cameron 1978, 1979a). Chloride uptake is linked with bicarbonate excretion and Krogh's (1938) hypothesis of chloride/bicarbonate exchange appears to hold for the crustaceans. There are numerous lines of evidence in support of this hypothesis, including a role for CA in providing bicarbonate for the exchange. The exchange mechanism is believed to be localized in the apical

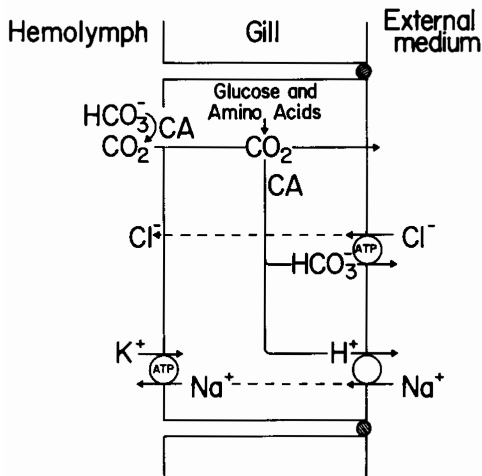

Fig. 1. A model for the distribution and function of carbonic anhydrase (CA) in crustacean gills

membrane (see Kirschner 1983). A bicarbonate ion-stimulated ATPase also appears to be involved in the process. A bicarbonate ATPase is present in two ion regulating species of crabs, *Uca minax* and *C. sapidus* (DePew and Towle 1979; Lee 1982). Both studies present strong evidence for ATPase activity in the plasma membrane fractions of gill homogenates. Bicarbonate ATPase activity increases by synthesis of more enzyme when *C. sapidus* is exposed to low salinity (Lee 1982), but no activity change is apparent in *U. minax* transferred to low salinity (DePew and Towle 1979). Thiocyanate inhibits the ATPase activity in vitro (DePew and Towle 1979; Lee 1982) and reduces chloride influx in vivo in *C. sapidus* (Cameron 1979a).

Carbonic anhydrase fits into this scheme by providing the Cl^-/HCO_3^- exchanger with bicarbonate from an intracellular pool of molecular CO_2. Two studies have demonstrated disruption of hemolymph Cl^- regulation in the decapod crustaceans maintained in dilute media and treated with the CA inhibitor acetazolamide (Burnett et al. 1981; Henry and Cameron 1983). The effects of acetazolamide treatment on unidirectional ion fluxes have been measured in crayfish (Ehrenfeld 1974) and freshwater blue crabs (Cameron 1979a). In both cases the influx of chloride ions increases accompanied by a greater increase in chloride efflux. These acetazolamide-related changes result in no net flux of chloride ions in the crayfish and a net efflux in the blue crab. Ehrenfeld (1974) suggested that these results could be explained if acetazolamide caused Cl^-/HCO_3^- exchange to become predominantly Cl^-/Cl^- exchange.

Acetazolamide treatment also has an effect on sodium ion regulation and sodium fluxes. However, sodium ion regulation is not affected to as great a degree as chloride ion regulation in *C. sapidus* (Henry and Cameron 1983). This is generally interpreted to mean that a significant fraction of the sodium uptake at the apical membrane is via a Na^+/H^+ or Na^+/NH_4^+ exchange. Protons for both types of exchange are, therefore, provided by the hydration reactions of CO_2 within the cell.

The evidence indicates that the low salinity adaptation process consists of the synthesis of more Na^+/K^+ ATPase and HCO_3^- ATPase (Lee 1982; Péqueux et al. 1984). The results of an experiment using blue crabs treated with acetazolamide and then transferred to low salinity (Henry and Cameron 1982a) are consistent with this suggestion. The survival of blue crabs treated in this manner is very low compared with the 100% survival of crabs acclimated to low salinity for at least 14 days and then treated with acetazolamide (Henry and Cameron 1983). In both cases sodium and chloride ion regulation is disrupted and bicarbonate and hydrogen ions are supplied to exchangers at a rate limited by the uncatalyzed hydration of a portion of the intracellular molecular CO_2 pool as a result of acetazolamide treatment. Clearly a large number of Cl^-/HCO_3^- exchangers working submaximally in the acclimated crab is an advantage over a small number working submaximally in the unacclimated crab.

An observation by Lee (1982) is worth mentioning in that it challenges this traditional view of the role of carbonic anhydrase in ionic regulation as espoused above. Lee (1982) observed that acetazolamide inhibited the bicarbonate-stimulated ATPase in the gills of blue crabs. This result suggests that the numerous observations made on ion transport using acetazolamide treatment have been ascribed erroneously to CA inhibition when they may have been due to a specific effect of the drug treatment on the Cl^-/HCO_3^- exchange mechanism. These observations are difficult to integrate into the current concept of CA function in ionic regulation. For the moment the difficulty

is mitigated by recognizing that the two concentrations of acetazolamide used in Lee's experiments were very high (4 and 5 m*M*). Maren (1977) has cautioned that using high concentrations (> 1 m*M*) of acetazolamide may result in nonspecific effects. Maren has also urged that other known inhibitors of CA, such as ethoxzolamide and methazolamide, should be used in physiological studies to pin down the specificity of action of CA inhibition. Unfortunately, this advice has not been followed by workers in this field. However, it is clear that the problem of a specific effect of acetazolamide on bicarbonate ATPase needs to be addressed experimentally.

In summary, intracellular CA is clearly implicated in the process of moving Na^+ and Cl^- into the cell across the apical membrane by exchange processes. The role of CA is the hydration of the intracellular molecular CO_2 pool to bicarbonate, which is used in the Cl^-/HCO_3^- exchange mechanism, and a hydrogen ion, which is used in the Na^+/H^+ or Na^+/NH_4^+ mechanism.

4 Carbon Dioxide Excretion

A role for gill CA in CO_2 excretion is supported by results of numerous investigations. We will review all the evidence and attempt to assemble a coherent picture of the enzymes functioning in crustacean gills which is consistent with the data. However, several investigators have challenged the idea that CA has any role in CO_2 excretion (Aldridge and Cameron 1979; Cameron 1979b; Henry and Cameron 1983). Since the issue is controversial, we will state what we perceive to be the main points of controversy and address these issues first. The controversy focuses on two points: (1) an acetazolamide-induced increase in hemolymph P_{CO_2} which should occur if CA were important to CO_2 excretion, and (2) the absence of a change in CO_2 excretion in whole animals after treatment with acetazolamide.

4.1 Does Hemolymph P_{CO_2} Increase when Carbonic Anhydrase is Inhibited?

In trying to resolve this question we will call upon data from all the publications concerning the acid-base responses to acetazolamide treatment in the decapods. Aldridge and Cameron (1979) measured hemolymph pH and total CO_2 concentrations in *C. sapidus* and found that there was no change in either variable, and therefore, no change in P_{CO_2}, for up to 6 h after crabs were injected with acetazolamide. However, Henry and Cameron (1983) working on the same species demonstrated that acetazolamide does have an effect on hemolymph acid-base status, but it takes about 12 h for the effect to be fully established. A similar situation was shown to occur in the red rock crab *Cancer productus* where maximal effects on hemolymph acid-base status occurred around 12 h after acetazolamide treatment (McMahon et al. 1984). Only four studies are available where acid-base variables were measured on animals at least 12 h after acetazolamide treatment. Randall and Wood (1981) were the first to report data on hemolymph P_{CO_2} in a land crab, *Cardisoma carnifex*, after acetazolamide treatment. They observed a significant increase in hemolymph P_{CO_2} as a result of the treatment (Table 1). A similar result was found in another terrestrial species, *Gecarcinus lateralis*

Table 1. Effect of 12 h acetazolamide treatment on hemolymph P_{CO_2}

Species	Control	Treatment	Reference
TERRESTRIAL			
Cardisoma carnifex	13	18	Randall and Wood (1981)
Gecarcinus lateralis	7.3	13.2	Henry and Cameron (1983)
AQUATIC			
Pachygrapsus crassipes			
Salinity = 43 ppt	2.9[a]	4.8[a]	Burnett et al. (1981)
Salinity = 35 ppt	2.8[a]	5.4[a]	
Salinity = 21 ppt	2.5[a]	5.2[a]	
Salinity = 13 ppt	3.3[a]	4.1[a]	
Callinectes sapidus			
Salinity = 30 ppt	2.2[a]	4.1[a]	Henry and Cameron (1983)
Salinity = 9 ppt	2.5	3.5	
Cancer productus			
Salinity = 31 ppt	1.3	4.5	McMahon et al. (1984)

[a] Calculated from pH and total CO_2 using constants reported by Truchot (1976)

(Henry and Cameron 1983). Henry and Cameron (1983) claim that in the fully aquatic species *C. sapidus* held in low salinity, hemolymph P_{CO_2} increases by a small, but statistically not significant, amount as a result of acetazolamide treatment. Although these authors did not measure hemolymph P_{CO_2} in this species acclimated to seawater, P_{CO_2} calculated from the pH and total CO_2 nearly doubles (Table 1). When hemolymph pH and total CO_2 data from another species, *Pachygrapsus crassipes*, are similarly analyzed, P_{CO_2} is shown to increase as a result of acetazolamide treatment at four different acclimation salinities (Table 1). Finally, hemolymph P_{CO_2}, measured directly and not calculated, more than doubled 12 h after *C. productus* were injected with acetazolamide (Table 1). Thus, the evidence seems to indicate overwhelmingly that CA inhibition results in an increase in hemolymph P_{CO_2} in the decapod crustaceans.

4.2 Acetazolamide Treatment Results in no Change in CO_2 Excretion

The effect of CA inhibition on whole animal CO_2 excretion has been measured on only two decapods, *G. lateralis* and *C. sapidus* (Henry and Cameron 1983). In both species acetazolamide treatment induces no change in either oxygen consumption or CO_2 production for up to 96 h. Given that hemolymph P_{CO_2} increases and that oxygen consumption remains constant when CA is inhibited, a sustained depression of CO_2 production is unlikely in the long run. The constancy of oxygen consumption can be taken to indicate that CO_2 production is unaffected by acetazolamide treatment. CO_2 moves across the gills at pretreatment rates driven by a larger molecular CO_2 gradient between hemolymph and the ambient medium. As discussed by Burnett (1984), yet another factor may be involved in that a large chemical disequilibrium between hemolymph bicarbonate and molecular CO_2 may exist as a result of acetazolamide treatment. Several studies have shown that CA activity exists in tissues other than the gills, e.g., muscle, heart, epithelial lining of the branchial chamber, and green

gland (Henry and Cameron 1982b; McMahon et al. 1984). It is possible that CA activity in these tissues normally serves to catalyze the hydration of metabolically produced CO_2, moving the hemolymph HCO_3^-/CO_2 system, which is 97%-98% HCO_3^- and 2%-3% CO_2, toward chemical equilibrium. Support for this hypothesis is found in the acetazolamide-induced pH disequilibria in perfused rabbit hind limb and liver, both of which contain CA activity (O'Brasky and Crandall 1980). Aldridge and Cameron (1979) have suggested that the hemolymph exists normally in disequilibrium favoring molecular CO_2 because of the lack of CA in the hemolymph and the relatively short transit times of hemolymph between the tissues and the gills. There is neither evidence for nor against the disequilibrium hypothesis in an untreated animal. However, if a hemolymph HCO_3^-/CO_2 disequilibrium were created or increased by acetazolamide treatment, then the molecular CO_2 gradients between hemolymph and the ambient medium would be even larger than the measurements indicate. Finally, it is possible that a transitory decrease in CO_2 excretion occurs soon after acetazolamide treatment as the P_{CO_2} difference across the gill increases. Henry and Cameron (1983) may not have detected such a decrease in CO_2 excretion, since their first measurements were 6 h after acetazolamide treatment.

4.3 Other Evidence for CO_2 Excretion

Using an isolated perfused gill from a crab which is an osmoconformer, Burnett (1984) has shown that $^{14}C-CO_2$ fluxes (label originating as both HCO_3^- and CO_2) across the gill decrease rapidly (within 15 min) when the gill is treated with acetazolamide. In this experiment the HCO_3^-/CO_2 system (both labeled and unlabeled pools) is in chemical equilibrium as the internal perfusate enters the gill, discounting the possibility that CO_2 excretion is facilitated by a disequilibrium favoring molecular CO_2.

The isolated perfused crab gill can be used to test aspects of a computer model of CO_2 excretion presented by Aldridge and Cameron (1979). One interesting point of the CO_2 excretion model is it predicts that CO_2 excretion is relatively unaffected by variations in hemolymph flow rate. This is so, according to the model, because most of the molecular CO_2 diffuses into the ambient medium during the first second or so it resides in the gill. Thereafter, molecular CO_2 is recruited from the large hemolymph bicarbonate pool at the slow, uncatalyzed rate. Thus, after the initial fast flux of CO_2, CO_2 excretion is rate limited by the dehydration reaction CO_2 in the hemolymph. We have recently performed experiments on isolated perfused crab gills *(Cancer anthonyi)* where we tested this aspect of the model directly by varying the residence time of the internal perfusate within the gill and measuring the efflux of ^{14}C label originating as bicarbonate and molecular CO_2 (in chemical equilibrium). These experiments were performed using procedures already described (Burnett 1984). We wish to report here for the first time that the flow rate of the internal perfusate through the gill has a profound effect upon the efflux of the label (Fig. 2). This is in contrast to the situation predicted by the model, but consistent with evidence presented so far on a role for CA in CO_2 excretion. Furthermore, when we treated the perfused gill with acetazolamide, CO_2 excretion decreased, as previously shown (Burnett 1984). However, acetazolamide treatment rendered CO_2 excretion across the gill insensitive to the flow rate of the internal perfusate (Fig. 2), a situation predicted by the model for the uncatalyzed inter-

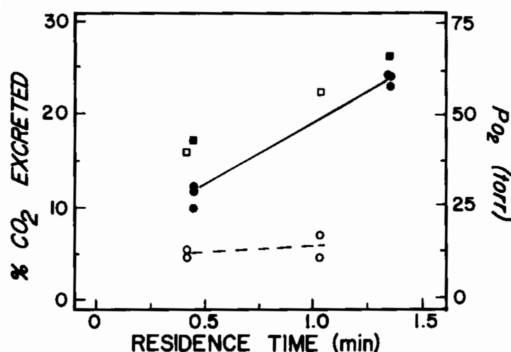

Fig. 2. The percent of ^{14}C label (as HCO_3^- and CO_2) excreted from the internal perfusate as a function of the residence time of the internal perfusate in the isolated perfused gill of *Cancer anthonyi* (*closed circles*). Residence time was calculated as gill hemolymph volume/flow rate of internal perfusate. The gill hemolymph volume was measured using the time course of the washout of ^3H-inulin from the hemolymph of the gill. The flow rate of the internal perfusate was controlled by perfusion pumps. Also shown is the effect of acetazolamide (4×10^{-4} *M*) treatment on the percent of ^{14}C label excreted (*open circles*). Values for P_{O_2} in the postgill internal perfusate are represented by *closed squares* (pre-acetazolamide treatment) and *open squares* (post-acetazolamide treatment). The variables for the internal perfusate are pH = 7.636, C_{CO_2} = 4.85 m*M*, P_{CO_2} = 2.2 torr, and pregill P_{O_2} = 7 torr

conversion of hemolymph bicarbonate and CO_2. A comparison of CO_2 efflux with oxygen uptake into the internal perfusate in this experiment confirms that the large decreases in CO_2 flux are due to a specific effect of CA inhibition and not an effect of acetazolamide on the degree or pattern of gill perfusion (Fig. 2). Oxygen uptake results in internal perfusate P_{O_2}s significantly elevated above the values entering the gill (5–10 torr). Control and acetazolamide-treated gills show no difference in their ability to take up oxygen at the internal perfusate flow rates tested.

5 A Model for CA Distribution and Function

A model for the distribution and function of gill CA must incorporate a role relating to both ionic regulation and CO_2 excretion. We suggest a model in which CA is localized both in the cell and on the cell surface (Fig. 1). The intracellular enzyme has a role in ionic regulation while the cell surface enzyme functions primarily in CO_2 excretion. Although direct evidence for the dual localization is unavailable, precedences for our placement of the enzyme are found in respiratory and ion transporting epithelia. Although we are currently conducting studies on the localization of CA in gills, we can only offer circumstantial evidence for its presence on the basal surface of the epithelium. Such a placement is the best model for incorporating the available data on both the distribution and multiple functioning of CA. In citing the following examples, we intend to show relevant, functional relationships in the localization of CA in other tissues.

The presence of CA in pulmonary tissues of mammals has attracted much attention recently (see for example Enns and Hill 1983). Pulmonary CA apparently facilitates the transport of CO_2 across two epithelial layers (the endothelial cells of capillaries and the epithelium of the lung) into the alveolar air space. Ryan et al. (1982) have demonstrated convincingly, using ferritin-labeled antibodies, that the CA is localizd on the surface of the endothelial cells, next to the blood. The positioning of the enzyme favors its access to the large blood bicarbonate pool which provides substrate for the dehydration of bicarbonate to the diffusible molecular CO_2. This system operates in conjunction with the well-known CA system in red blood cells to remove CO_2 from the blood during its relatively short transit time through the lung. A similar positioning of CA along the basal membrane of the crustacean gill serves a similar respiratory function, with a major difference being the lack of CA in the circulating hemolymph.

The ion transporting function of the crustacean gill is, of course, not found in the example of the mammalian lung. One of the best studied examples of the involvement of CA in transporting epithelia is the proximal convoluted tubule of the vertebrate nephron. CO_2 is transported ultimately from the lumen of the tubule to the serosal side of the epithelium as bicarbonate. Although the presence of CA on the brush border has already been demonstrated, a recent important finding establishes that CA has access to the fluid in the lumen of the tubule (Lucci et al. 1983). This is important in this instance both because CA has access to the extracellular bicarbonate pool and because a separate intracellular CA also exists. The brush border CA functions in dehydrating the luminal bicarbonate pool to diffusible CO_2 much the same way the crustacean gill CA dehydrates the hemolymph bicarbonate pool. Intracellular CA then acts to hydrate CO_2 in both the proximal tubule and the gill.

The main point of the above discussion is that similar strategies have been observed in tissues which have similar respiratory and ion transport functions. The existence of an extracellular or cell surface CA in crustaceans has yet to be shown, but there is a suggestion that an extracellular CA plays a role in shell calcification in crabs (Giraud 1981).

Another aspect of the model illustrates that the gill epithelial cells may be metabolically quite active. The intracellular molecular CO_2 pool consists not only of CO_2 passing from the hemolymph to the ambient medium, but also CO_2 generated endogenously. Gill tissues have been shown to be metabolically active, producing CO_2 from sources, such as alanine, arginine, glutamate, glycine, proline, and glucose (Pressley and Graves 1983). Such has also been shown to be the case in isolated perfused gills of the osmoconforming *C. anthonyi* which oxidize glucose (Burnett and Grieshaber, unpublished results). In this case, the addition of $(U)^{14}C$-glucose to the internal perfusate resulted in unidirectional fluxes of $^{14}C-CO_2$ from the gill epithelial cells into the hemolymph space and into the surrounding seawater. Thus, CO_2 passing from the hemolymph to the ambient medium must do so through metabolically active tissues where intracellular P_{CO_2} cannot be negligible. In order to have a net flux of CO_2 from hemolymph to ambient medium, the P_{CO_2} of the hemolymph must be maintained at least above that of the epithelial cell itself.

Any model which deals with CO_2 excretion across gills such as the one advanced by Aldridge and Cameron (1979) must account for the higher than ambient P_{CO_2} of the epithelial cell. While the Aldridge and Cameron (1979) model has played an im-

portant role in stimulating a rigorous quantitative approach to the kinetics of CO_2 excretion across gill tissues, it must now be modified to account for the requirement of the maintenance of a higher overall hemolymph P_{CO_2} to explain physiological rates of CO_2 excretion. We suggest that the maintenance of a P_{CO_2} gradient favorable for CO_2 excretion requires a substantial recruitment of molecular CO_2 from the hemolymph bicarbonate pool. Neither the available quantities of molecular CO_2 nor its recruitment from hemolymph bicarbonate at the uncatalyzed rate can account for the quantity of CO_2 actually excreted even in the absence of CO_2 produced within the gill and assuming a molecular CO_2 concentration of zero in the ambient medium (Burnett 1984). In order for the simulation to work, the hemolymph HCO_3^-/CO_2 system must be in disequilibrium favoring molecular CO_2 or CA must be active in recruiting from the hemolymph bicarbonate pool or both. As pointed out by Burnett (1984), there is neither evidence for nor against the disequilibrium hypothesis. However, as we have shown in this article, there is abundant evidence which supports the action of the enzyme.

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