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Biochemistry, Localization, and Physiology of Carbonic Anhydrase in the Gills of Euryhaline Crabs

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ABSTRACT Carbonic anhydrase, found at high levels in crustacean gill, is capable of catalyzing the formation of CO_2 in the forward reaction or the production of HCO_3^- in the reverse reaction. Thus the enzyme may participate in both respiratory and ion regulatory functions. Its subcellular distribution in crab gill epithelial cells suggests that a large proportion of the enzyme is membrane-bound, where it would be available to pharmacological treatment with membrane-impermeable carbonic anhydrase inhibitors. These studies suggest that the enzyme plays a central role in CO_2 excretion. Treatment with the permeable inhibitor acetazolamide affects osmoregulation and ion transport in addition to CO_2 excretion, suggesting that the cytosolic form of carbonic anhydrase may provide HCO_3^- for Cl^-/HCO_3^- exchange and H^+ for Na^+/H^+ exchange across the gill epithelium.

Carbonic anhydrase (CA) (EC 4-2-1-1) is a widespread enzyme within animal tissues which reversibly catalyzes the establishment of the equilibrium between carbon dioxide and the bicarbonate system:

$$CO_2 + H_2O \overset{CA}{\rightleftharpoons} HCO_3^- + H^+. \quad (1)$$

The enzyme accelerates the hydration of CO_2 and the dehydration of HCO_3^-, reactions which also, but with much reduced rates, proceed in absence of the enzyme. CA thus catalyzes the rapid conversion of CO_2 to HCO_3^- and H^+, charged chemical species of essential significance for processes of ion transport, acid-base equilibrium, and calcification. Reversibly, the small neutral molecule CO_2 is formed, a gas which is highly water soluble and easily diffusible across cellular membranes.

Since its first description by Meldrum and Roughton ('32) the CA of mammals and other vertebrates has been investigated intensely (Maren, '67, '80; Crandall and O'Brasky, '78; Henry, '84; Bruns et al., '86; Gros et al., '88). Besides its predominant role in respiratory processes, the enzyme participates in a remarkable multitude of functions.

Highest activities have been measured in erythrocytes. Carbonic anhydrase also fulfills specific functions in the stomach (Davenport, '39; Maren, '67; Carter, '72; Tashian, '77), in the lung (Crandall and O'Brasky, '78), in the kidney (Maren, '67; Dobyan and Bulger, '82), in the liver (Gros et al., '88; Walsh et al., '89), in muscle (Holmes, '77; Bruns et al., '86), and in the eye (Bill, '75; Zadunaisky, '78; Helbig et al., '89).

Activities of carbonic anhydrase were also found in erythrocytes, the swimming bladder, the kidney, and the gills (Maren, '67; Haswell et al., '80) of fishes. In fish gills, CA is assumed to play a role in excretion of CO_2 and regulation of blood osmolality and acid-base balance (Perry and Laurent, '90). It is supposed that the enzyme participates in respiratory processes by facilitating the diffusion of CO_2 from blood to ambient medium (Dimberg et al., '81; Rahim et al., '88). Several reports take into consideration a role of branchial CA in the process of osmoregulation (Maetz and Garcia Romeau, '64; Maren, '67; Maetz, '74; Girard and Istin, '75) and acid-base equilibrium (Dimberg and Höglund, '87; Swenson and Maren, '87; Conley and Mallatt, '88). The enzyme is considered to provide the counterions H^+ and HCO_3^- for the apical ion exchange system (Na^+/H^+ and Cl^-/HCO_3^-) which links ion uptake and acid-base regulation in the gills of freshwater fishes (Maetz and Garcia Romeau, '64; Kerstetter et al., '70; Maetz, '71). As reported by McDonald and Prior ('88), the presence of the ion exchangers has not been clarified to date. These authors suppose that apical exchange mechanisms play a rather limited role in excretion of acid equivalents across fish gills.

CARBONIC ANHYDRASE IN INVERTEBRATE TISSUES

Activities of carbonic anhydrase have been found in the tissues of various invertebrate species. The

presence of the enzyme has been reported in coelenterates (Brinkman, '33; Goreau, '59; Aronova et al., '86; Kingsley and Watabe, '87), echinoderms and annelids (Brinkman, '33), molluscs (Brinkman, '33; Ferguson et al., '37; Florkin and de Marchin, '41; Freeman and Wilbur, '48; Maren, '67; Addink, '71; Wilbur, '72; Nielsen and Frieden, '72; Wheeler, '75; Boer and Witteveen, '80; Aronova et al., '86; Henry, '87a; Drews, '90), and arthropods (Ferguson et al., '37; Mahuf, '40; Anderson and March, '56; Costlow, '59; Dresco-Derouet, '65; Maren, '67; Turquier, '68; Turbeck and Foder, '70; Stobbart, '71; Johnston and Jungreis, '79; Henry, '84, '86, '88b; Darlington et al., '84; Burnett et al., '81, '85; Aronova et al., '86).

Henry ('87a) investigated the blood cells of several invertebrate species for activity of CA. Annelid red blood cells and sipunculid pink blood cells possessed significant levels of activity. In molluscan blood cells no activity was detected. The hemolymph of the oyster *Crassostrea virginica* contained an extracellular CA with affinities for acetazolamide and ethoxzolamide comparable to vertebrate CA II.

In comparison with vertebrates, information about occurrence and functioning of CA in invertebrates is fragmentary. Among the physiological roles suggested for invertebrate CA is its respiratory function and its role in facilitating the processes of calcification. Participation of the enzyme in the latter process was reported to occur in sponges (Jones and Kedger, '86), coelenterates (Kingsley and Watabe, '87), molluscs (Wilbur and Saleuddin, '83), crustaceans (Giraud, '81; Cameron and Wood, '85; Henry and Kormanik, '85), and echinoderms (Chen and Lawrence, '86; Donachy et al., '90). The influence of acetazolamide on the processes of mineralization was investigated in coelenterates, molluscs, crustaceans, and echinoderms (Wilbur and Jodrey, '55; Costlow, '59; Goreau, '59; Mitsunaga et al., '86). Summarizing these reports, the enzyme seems to be indispensable for the calcification process but may facilitate parts of it.

CARBONIC ANHYDRASE IN CRUSTACEAN GILLS

Significant specific activities of CA have been reported in respiratory and excretory organs of crustaceans, such as the lungs (formed from the lining of the branchial chamber) of terrestrial crabs and the renal organ of crayfishes. Highest activities were found in gills. Crustacean gills are multifunctional organs playing a central role in respiration, ion regulation, nitrogen excretion, and acid-base equilibrium. These functions are considered to be highly intercorrelated.

With regard to the catalyzed reaction (1), two major roles of branchial CA have been proposed in decapod crustaceans. Catalysis of CO_2 formation would facilitate excretion of CO_2, and the reverse reaction producing HCO_3^- would facilitate osmoregulatory processes involved in ion transport processes.

This work summarizes the pertinent information on CA in crustacean gills and describes the results of a series of experiments on the biochemistry, subcellular localization, and the physiological function of branchial CA in the shore crab *Carcinus maenas*. The potential roles of the enzyme in respiration and active osmoregulatory ion uptake are considered with special emphasis.

ORGAN DISTRIBUTION AND CELLULAR LOCALIZATION OF CARBONIC ANHYDRASE IN CRUSTACEANS

Distribution of CA activity has been examined in the tissues of some decapod crustaceans, among them *Pachygrapsus crassipes* (Burnett et al., '81), *Cardisoma carnifex* (Randall and Wood, '81), *Callinectes sapidus, Libinia emarginata, Gecarcinus lateralis, Cardisoma guanhumi* (Henry and Cameron, '82), *Cancer productus* (McMahon et al., '84), *Pacifastacus leniusculus* (Wheatly and Henry, '87), *Birgus latro* (Morris and Greenaway, '90), *Carcinus maenas* (Böttcher et al., '90a), and *Eriocheir sinensis* (Böttcher and Siebers, '90). So far, investigated CA activity was never found in the hemolymph. In muscle, heart, digestive gland, male gonads, and hypodermis CA activity was very low or not detectable. In the antennal gland of the crayfish *P. leniusculus*, high and salinity-dependent activities of CA were found by Wheatly and Henry ('87) and Henry and Wheatly ('88), a result discussed with respect to the ability of this freshwater species to produce hypoosmotic urine. Recently, Morris and Greenaway ('90) found CA activity in the membrane-bound fraction of the branchial chamber lining (lung) of the terrestrial crab *B. latro* and considered it to be correlated with CO_2 excretion by the lungs.

Among the organs tested, highest CA activities were found in the gills. In the euryhaline crabs *C. maenas* and *C. sapidus*, which hyperregulate hemolymph osmolalities when exposed to dilute media, CA activity of posterior gills exceeded that of anterior gills significantly (Henry and Cameron, '82; Böttcher et al., '90a). Following acclimation to low

salinities, CA activity of posterior gills of these crabs increased about 2-fold compared with seawater controls (Fig. 1) (Böttcher et al., '90a). The same salinity-dependent distribution of CA activity was found in the gills of the euryhaline chinese crab *E. sinensis* (Böttcher and Siebers, '90).

When acclimated to a salinity of 950 mOsm, branchial CA activity of the semiterrestrial crab *C. guanhumi* was distributed comparably with the CA activity in the gills of *C. sapidus* when exposed to low salinity (Henry and Cameron, '82). Like *C. sapidus,* this crab is an osmoregulator but it regulates hemolymph osmolality below that of the ambient medium. Following the study of Henry and Cameron ('82), CA activity of the posterior gills of the terrestrial crab *G. lateralis* was only slightly increased compared with the anterior gills.

The limnic crustacean *P. leniusculus* is isosmotic in a salinity of 475 mOsm. It hyperregulates hemolymph osmolality when exposed to fresh water and hyporegulates when maintained in 750 mOsm. In this crustacean no difference between CA activities of anterior and posterior gills was found, and CA activity was increased only in the gills of crayfishes exposed to fresh water (Wheatly and Henry, '87). Branchial CA activity of the stenohaline marine osmoconformer *L. emarginata* was low and homogeneously distributed over the gills (Henry and Cameron, '82). McMahon et al. ('84) examined branchial CA activity of the stenohaline marine crab *C. productus* and found no significant differences between anterior and posterior gills. Following these studies, increased CA activities of posterior compared with anterior gills seem to be characteristic of euryhaline aquatic crabs like *C. sapidus, C. maenas,* and *E. sinensis,* as well as of semiterrestrial (*C. guanhumi*) and terrestrial (*G. lateralis*) species. Homogenous CA activities in all gill pairs were found in stenohaline marine (*L. emarginata, C. productus*) and freshwater species (*P. leniusculus*).

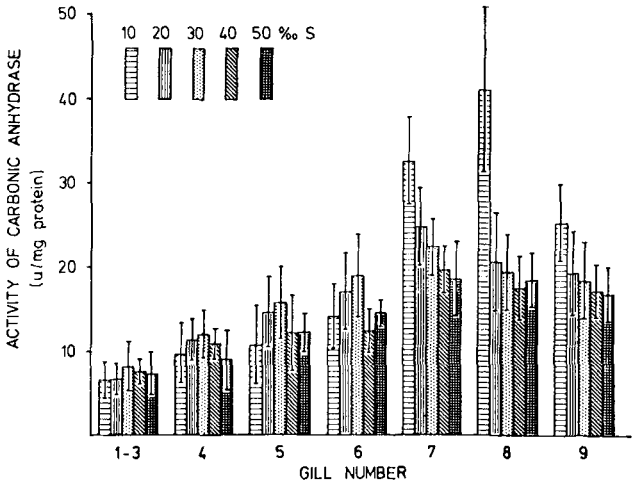

Fig. 1. *Carcinus maenas.* Specific activities of carbonic anhydrase in the gills of shore crabs acclimated to salinities between 10 and 50 ‰ S. Homogenates were prepared with 1% Triton X-100. Data are means ± standard deviations obtained from a sample size of the gills of 6 crabs. (From Böttcher et al., '90a.)

In order to assess the physiological role of branchial CA, the subcellular localization of the enzyme within the epithelial cells of crustacean gills was investigated. Burnett et al. ('81) centrifuged gill homogenates of crabs *P. crassipes* at 100,000*g* and treated the resuspended pellets with 1% of the detergent Triton X-100. The suspension was centrifuged again. The authors found all of the CA activity in the Triton-free supernatant of the first centrifugation and no detectable activity in the Triton-containing supernatant of the second centrifugation or the pellet. Since membrane-associated branchial CA was not found, it was deduced that all enzyme was soluble. Using differential centrifugations, Randall and Wood ('81) found the branchial CA of the land crab *C. carnifex* in cytoplasm as well as in microsomes. This is the first report of bound CA in crustacean gills. Henry and Cameron ('83) discussed the possibility of CA activity partially bound to basolateral membranes to facilitate CO_2 excretion across the gill epithelium, but considered it unlikely since hemolymph of *C. sapidus* contains about 250 mM chloride ions which are known to inhibit CA activity (Maren, '67; Henry and Cameron, '82). Burnett et al. ('85) first described a model for the distribution and function of CA in crustacean gills, including a soluble form of the enzyme in cytoplasm providing the counterions for apical Cl^-/HCO_3^- and Na^+/H^+ exchange and a basolateral, membrane-associated form facilitating CO_2 excretion by catalyzing the dehydration of the large hemolymph bicarbonate pool to the more diffusible molecular CO_2.

Application of an impermeable dextran-bound CA inhibitor to the basolateral side of isolated perfused gills of the crab *C. productus* significantly reduced CO_2 efflux from the internal to the external side (Burnett and McMahon, '85). The authors discussed their finding with respect to a CA fraction localized on the luminal surface of the basal membrane with its active site facing the lumen of the hemolymph space and facilitating CO_2 excretion. Henry ('87b) injected the membrane-impermeant CA inhibitor quaternary ammonium sulfanilamide (QAS) into the hemolymph of *C. sapidus* and reported an increase in the partial pressure of CO_2,

a result supporting the assumption of the presence of CA on the basal membrane of the gill cells.

Using differential and density gradient centrifugations, Henry ('88a) showed that both the anterior and posterior gills of *C. sapidus* acclimated to 35 or 12 ‰ SS contained cytoplasmic as well as membrane-bound CA activity. In the gills of crabs acclimated to high salinity, the author found approximately equal amounts of CA activity in cytoplasmic and membrane fractions. In anterior gills these activities were not altered by environmental salinity. In posterior gills membrane CA activity increased between 50 and 100%, and cytoplasmic CA activity was enhanced 20-fold when crabs were acclimated to 12 ‰ S. Determination of enzyme concentrations in posterior gills of crabs acclimated to 12 ‰ S revealed that 6.5% of the CA was bound and 93.5% was soluble (Henry, '88a).

In gill homogenates of *C. maenas,* employment of Triton X-100 resulted in about 2-fold increased activities of CA compared with homogenates prepared without detergent (Böttcher et al., '90a). This finding implies at least partial association of CA to cellular membranous structures. Centrifugation of gill homogenates of the shore crab *C. maenas* at 100,000*g* showed the presence of cytoplasmic and bound CA activity in the gills (Fig. 2). In the anterior gills of crabs acclimated to 10 ‰ S, bound CA activity amounted to ca. 90% and approximately 10% were found in the supernatants (Böttcher, '91). In posterior gills of *C. maenas* acclimated to 30 ‰ S, about 94% of CA activity was bound (Böttcher et al., '90a). In crabs acclimated to a salinity of 10 ‰, total CA of posterior gills consisted of 20 to 30% soluble and about 70 to 80% bound activity (Böttcher et al., '90a,b). The same proportions were found in the gills of the terrestrial crab *B. latro* (Morris and Greenaway, '90).

Experiments using 100,000*g* centrifugation of gill homogenates of crabs acclimated to 10 ‰ S show that proportions of soluble and particulate CA can differ considerably. Treatment of gill homogenate with Triton X-114 (a detergent that separates in an aqueous and a detergent phase above 20°C), according to Bordier ('81) and Wetzel and Gros ('90), resulted in only 11 to 13% soluble CA activity (unpublished data). Probably, the gills of *Carcinus* do not contain any cytoplasmic CA since the bulk of CA activity was found in the detergent phase.

Subcellular localization of branchial CA of *C. maenas* was investigated by Böttcher et al. ('90a,b) by means of density gradient centrifugations (Fig. 3). After centrifugation of a 7,500*g* supernatant of gill homogenates in a sucrose gradient (10 to 40%),

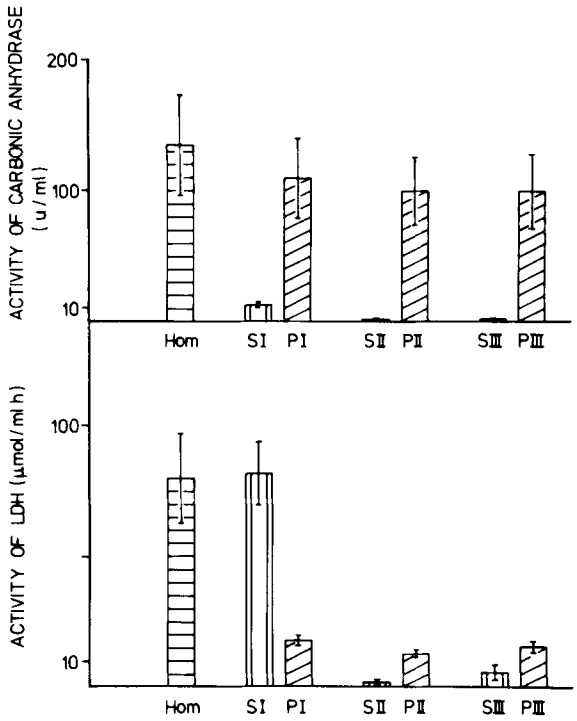

Fig. 2. *Carcinus maenas.* Activities of carbonic anhydrase and lactate dehydrogenase in homogenates, supernatants, and pellets obtained from 100,000*g* centrifugations. Prior to centrifugation the resuspended pellets were homogenized and sonicated. Homogenates were prepared from posterior gills (No. 7–9) of shore crabs acclimated to a salinity of 10 ‰ S. Data represent means ± standard deviations obtained from 3 experiments. (From Böttcher et al., '90b.)

two peaks of CA activity were determined. One of them was found in the fractions of the gradient characterized by high activities of LDH, an exclusively soluble enzyme. The other peak of CA activity was accompanied by high activities of Na-K-ATPase, a marker enzyme of basolateral plasma membranes. Density gradient centrifugation of a 7,500*g* pellet clearly separated mitochondria from plasma membranes. Using the mitochondrial marker cytochrome oxidase potential mitochondrial presence of branchial CA could be excluded. Rehomogenization and/or sonication of 100,000*g* pellets of gill homogenates did not result in liberation of enzyme activity into the supernatant after repeated centrifugations (Fig. 2) (Böttcher et al., '90b). The assumption that membrane-associated CA seemed to be bound integrally into membranes was published by Henry ('88a) after freeze-thawing and sonicating density gradient fractions containing the bound branchial CA of *C. sapidus.*

The presence of CA within the fractions of the density gradient exhibiting Na-K-ATPase activities (Henry, '88a; Böttcher et al., '90a,b) implies that

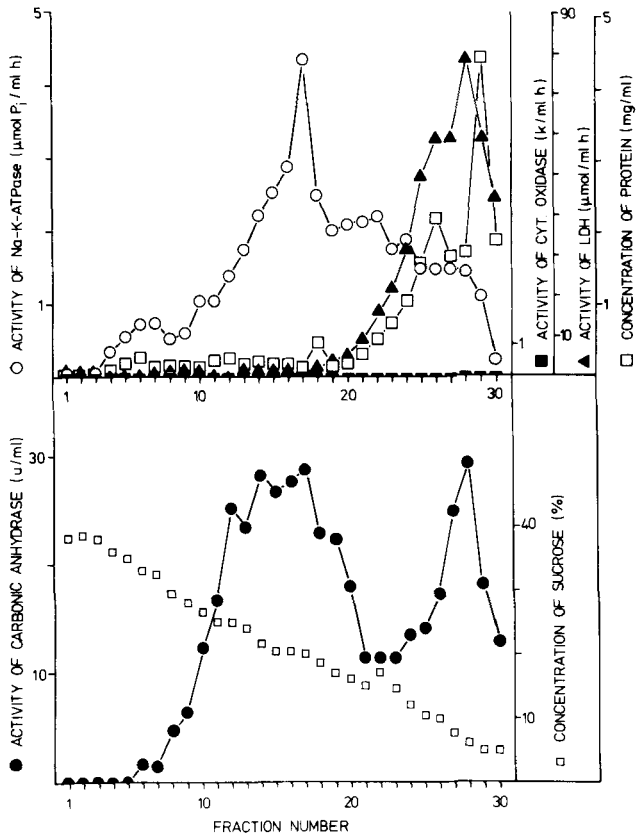

Fig. 3. *Carcinus maenas*. Activities of branchial carbonic anhydrase, Na-K-ATPase, lactate dehydrogenase, and protein concentrations in the fractions obtained from centrifugation of a 7,500*g* supernatant in a sucrose density gradient. A total of 6 posterior gills (No. 7–9) taken from 3 shore crabs acclimated to a salinity of 10 ‰ S were homogenized with homogenizing buffer (250 mM sucrose, 20 mM Tris/HCl, pH 7.6). The 750*g* supernatant was centrifuged at 7,500*g*. The resulting supernatant was layered on the top of a 10–40% (w/w) linear sucrose density gradient. (From Böttcher et al., '90a.)

bound CA is associated with basolateral plasma membranes. Due to the lack of marker enzymes, its presence in other membranes (e.g., apical membranes or endoplasmic reticulum) cannot be excluded.

BIOCHEMISTRY OF BRANCHIAL CA

Though several crustaceans have been investigated with respect to distribution and localization of branchial CA, little is known about the biochemical characteristics of the enzyme. In the following we summarize some data about the enzyme's inhibition by aromatic sulfonamides and anions, effects of detergents, the influence of dithiothreitol (DTT), and stability.

Henry ('88a) investigated some properties of the cytoplasmic and the membrane-bound branchial CA of *C. sapidus*. Treating membrane-bound CA (obtained by density gradient centrifugation) with 1% of the detergent sodium dodecyl sulfate (SDS), approximately 90% of activity was lost. Cytoplasmic CA activity was only slightly reduced by this procedure. The author supposed that bound CA activity depends on the integrity of the membranous environment. Böttcher et al. ('90a) found that the whole pool of branchial CA activity was lost by treatment with 1% SDS, a detergent known as a potent denaturing agent of proteins. Whitney and Briggle ('82) reported remarkable stability of membrane-associated CA of vertebrate lungs in solutions containing SDS. Particulate CA of human kidney was also stable in 3 to 4% SDS for about 24 h (McKinley and Whitney, '76).

Stability of both the cytoplasmic and the membrane-bound fractions of CA of gills of *C. sapidus* was investigated in controls and in the presence of 10 mM of the reducing agent DTT over a period of 7 days at a temperature of 5°C (Henry, '88a). Independently of the use of DTT, activities of both CA fractions remained rather stable. The author concluded that crustacean CA does not possess a high sulfhydryl content. Total branchial CA activity of *C. maenas* was remarkably stable when stored in the presence of 1% Triton X-100 at 0°C for 24 h and at −18 or −75°C for 28 d (Böttcher et al., '90a).

Branchial CA activity of two crab species, *C. sapidus* and *G. lateralis*, was dependent on pH and temperature (Henry and Cameron, '82). Optimum temperature was about 25°C. Activity was reduced with increasing pH from 6.8 to 8.0 independently of the temperature applied.

Aromatic sulfonamides are the strongest and most selective inhibitors of animal and bacterial CA (Wyeth and Prince, '77). Total CA activity of posterior gills of *C. maenas* was completely inhibited by 10^{-6} M acetazolamide or methazolamide (Fig. 4) (Böttcher et al., '90a). CA in supernatants of gill tissue of *P. crassipes* were quantitatively inhibited by 2×10^{-4} M AZ (Burnett et al., '81). An inhibitor concentration of 5×10^{-5} M reduced CA activity only partially.

Table 1 shows the inhibition constants of soluble and membrane-bound branchial CA of *C. sapidus* and *C. maenas* for some sulfonamides determined by the plot of Easson and Stedman ('37). Inhibition constants of soluble CA do not differ significantly from those of bound CA. This fact and the low

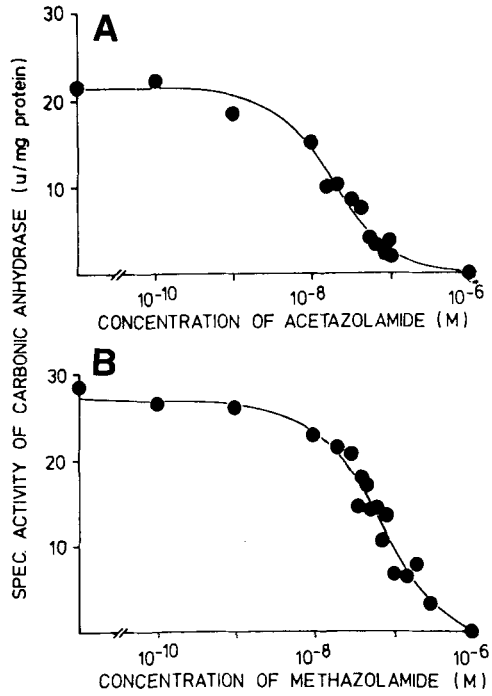

Fig. 4. *Carcinus maenas*. Activity of branchial carbonic anhydrase in relation to the sulfonamide inhibitors acetazolamide (**A**) and methazolamide (**B**). Homogenates were prepared from posterior gills (No. 8) acclimated to a salinity of 10 ‰ S. (From Böttcher et al., '90a.)

amount of supernatant CA in *Carcinus* gills support the recently developed assumption that there exists only a bound form of CA. Data range between 10^{-8} and 10^{-10} M showing similarity to vertebrate CA II in this respect. Inhibition constants of several bound carbonic anhydrases of vertebrate tissues are of comparable magnitude (Maren, '80; Whitney and Briggle, '82; Bruns et al., '86; Wistrand and Knuuttila, '89; Wetzel and Gros, '90).

TABLE 1. Inhibition constants of cytoplasmic and membrane-bound branchial carbonic anhydrase of the crabs Callinectes sapidus *and* Carcinus maenas *for acetazolamide (AZ), methazolamide (MZ), and ethoxzolamide (EZ) determined by the plot of Easson and Stedman ('37)*

	Inhibition constants (M)		
	AZ	MZ	EZ
Cytoplasmic CA:			
Callinectes sapidus[1]	$7.8 \cdot 10^{-9}$		$1.7 \cdot 10^{-9}$
Carcinus maenas[2]	$2.7 \cdot 10^{-9}$	$5.2 \cdot 10^{-9}$	$2.1 \cdot 10^{-10}$
Bound CA:			
Callinectes sapidus[1]	$6.7 \cdot 10^{-9}$		
Carcinus maenas[2]	$1.1 \cdot 10^{-8}$	$1.9 \cdot 10^{-9}$	10^{-9}

[1]Henry ('88a).
[2]Böttcher ('91).

Henry and Cameron ('82) treated branchial CA of *C. sapidus* and *G. lateralis* with NaCl concentrations ranging from 0 to 500 mM. Activity was decreased in a linear mode by increasing salt concentrations; at 500 mM NaCl activity was reduced to 35% of controls. In contrast, branchial CA activities of *C. maenas* were insensitive to 500 mM NaCl (unpublished results).

Unpublished results obtained in our laboratory suggest that the membrane-bound enzyme of the gills of *Carcinus* has a molecular weight of approximately 36 kDa, typical of kidney or lung membrane-bound carbonic anhydrases of vertebrates (Wistrand and Knuuttila, '89; Zhu and Sly, '90). This molecular weight clearly differs from the molecular weights of soluble isoenzymes amounting to ca. 30 kDa. More than 50% of the bound enzyme is liberated by phospholipase C, indicating that a considerable portion of this CA is membrane-anchored by phosphatidylinositol-linkage (Böttcher et al., unpublished).

PHYSIOLOGICAL FUNCTIONS OF CARBONIC ANHYDRASE IN CRUSTACEAN GILLS

Because the substrates and the products of the hydration/dehydration reaction are a diffusible gas and charged ions, it has been postulated that CA can be involved in respiratory gas exchange, ion transport, and acid-base regulation and may thus play a crucial role in these processes all occurring in the crustacean gill (Henry, '87b; '88b). The functions of branchial CA in respiration and ion transport are discussed separately. The enzyme's role in acid-base regulation, which is an integral feature of every CA reaction, is discussed within the considerations of the respiratory and ion transport functions.

Some properties of the catalyzed reaction and its components make assumptions on the physiological role of CA difficult. Substrates and products are interconverted also nonenzymatically, and the direction of the catalyzed reaction in vivo is difficult to determine because changes in the concentration of protons may be attenuated due to buffering capacities of physiological fluids. Therefore, most assumptions on the role of CA in decapods have been derived from the effects of CA inhibitors after various periods of application on ion concentrations, partial pressures of CO_2, and/or pH in hemolymph. In order to measure transepithelial fluxes of ions and inorganic carbon in controls and in the presence of specific CA inhibitors, isolated perfused gill preparations have been employed.

The respiratory role of branchial carbonic anhydrase

The respiratory organs of various kinds of terrestrial and aquatic animals all possess carbonic anhydrase. It was suggested by Burnett ('84) that the ubiquity of the enzyme in respiratory organs is best explained by its facility in mobilizing bicarbonate from blood or hemolymph into the respiratory epithelium as CO_2, from where it is finally excreted. With respect to the above mentioned difficulties to define the physiological roles of branchial CA, publications dealing with the assumed respiratory function of the enzyme are contradictory.

It has been concluded that in crab gills CA is involved in ion transfer rather than in CO_2 excretion because changes of the concentrations of CO_2 following AZ treatment were small or not observed (Aldridge and Cameron, '79; Cameron, '79; Randall and Wood, '81). Aldridge and Cameron ('79) injected acetazolamide into the hemolymph space of the crab *Callinectes sapidus* to obtain a final circulating concentration of 0.67 mg $\times$ ml^{-1}. Since the inhibitor did not affect hemolymph pH and CO_2 concentrations, the authors concluded that CA is probably not significantly involved in CO_2 excretion. Comparable results were obtained by Randall and Wood ('81), who inhibited carbonic anhydrase in the land crab *Cardisoma carnifex* by intravascular injection of 50 mg $\times$ kg^{-1} AZ. With regard to the resulting small increase in the partial pressure of blood CO_2, the role of CA in CO_2 excretion was considered as limited.

In contrast to these results, Burnett and coworkers ('81) found that in the crab *Pachygrapsus crassipes* the enzyme plays a role in hemolymph CO_2 regulation. AZ (2×10^{-4} M) added to the crab's ambient seawater equilibrated with the hemolymph within one h. The effects observed seemed to be confined specifically to crabs kept in dilute media. Hemolymph CO_2 levels increased slightly in higher and clearly in lower salinities.

Using isolated perfused gills of osmoconforming crabs *Cancer anthonyi* Burnett et al. ('81) demonstrated that branchial CA facilitates transgill CO_2 excretion. The authors followed the disappearance of $H^{14}CO_3^-$ and $^{14}CO_2$ from the internal perfusate in controls and in the presence of 4×10^{-4} M internally applied AZ or 0.4 mg $\times$ ml^{-1} commercial bovine erythrocyte CA. While AZ greatly decreased CO_2 excretion rates, addition of CA resulted in a high and significant increase. The author suggested two locations for crab gill CA. The enzyme should be located on or near the basal membrane where it would have ready access to the hemolymph bicarbonate pool, a localization consistent with its role in facilitating the transfer of inorganic carbon into the epithelial cell. An additional intracellular CA was assumed to utilize CO_2 for purposes of acid-base and ionic regulation.

The assumption of a respiratory role of branchial CA was confirmed by Burnett and McMahon ('85) using a dextran-bound membrane-impermeable inhibitor (DBI) of CA, which was synthesized of the glutaryl analog of 2-succinylamido-1,3,4-thiodiazole-5-sulfonamide having a molecular weight of 5,000–6,000. The DBI inhibited the CA in gill homogenates of the crab *Cancer productus* with an I_{50} of 0.165 $\mu g \times ml^{-1}$. Applied in the internal perfusate of an isolated perfused gill it did not penetrate across the epithelium into the external bath, and at a concentration of 1.25 $\mu g \times ml^{-1}$ inhibited the liberation of $H^{14}CO_3^-$ from perfusate to bath nearly completely.

Using isolated perfused gills of shore crabs *C. maenas*, Böttcher et al. ('91) showed that the excretion of inorganic carbon across the branchial epithelium depended in a saturable mode on the magnitude of the HCO_3^- gradient employed, a finding suggesting the participation of at least one rate-limiting transport protein in this process. In the presence of an HCO_3^- gradient of 6 mM directed from the internal perfusate to the external bath, unidirectional efflux of total inorganic carbon (measured by means of radiolabeled HCO_3^-) was reduced by nearly 80% following the application of 10^{-4} M external or 10^{-4}, 5×10^{-4}, and 10^{-3} M internal AZ (Fig. 5).

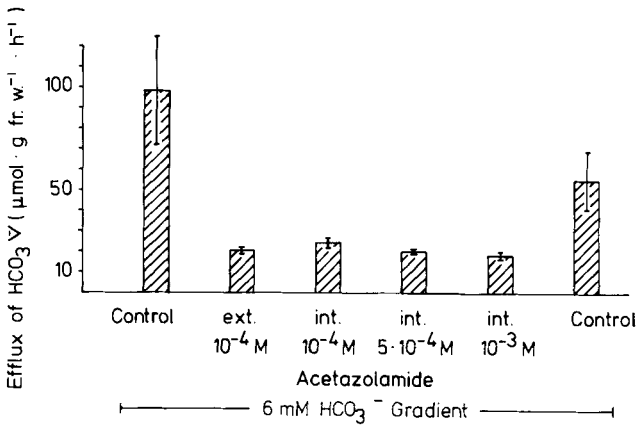

Fig. 5. *Carcinus maenas*. Bicarbonate efflux across isolated perfused posterior gills in relation to a 6 mM gradient of HCO_3^- directed from perfusate to bath. The gills were treated by externally (10^{-4} M) and internally (10^{-4} M, 5×10^{-4} M, and 10^{-3} M) applied acetazolamide, which was subsequently removed. Data represent means ± standard deviations obtained from a sample size of 4 gills. (From Böttcher et al., '91.)

These findings correlate with the work by Henry ('87b), who injected the membrane-impermeable CA inhibitor QAS (quaternary sulfanilamide) into the hemolymph space of the crab *Callinectes sapidus*, maintained at 12 ‰ S (350 mOsm) to reach a hemolymph concentration of 1 and 10 mM QAS. The injection resulted in a rapid (0.5 h) and temporary (up to 1 d) development of a respiratory acidosis with a rise of the partial pressure of CO_2 to 2 torr, lowering of pH by 2.5 units, and increase of total CO_2 by 2 mM. The effects were assumed to result from inhibitions of a CA associated with the basal membrane which catalyzes the conversion of hemolymph bicarbonate to CO_2.

The potential role of branchial CA in ion transport

It has been suggested that in crustaceans branchial carbonic anhydrase plays a major role in the processes of osmoregulatory ion uptake. In order to show the hypothetical relation between CA activity and osmotic regulation, the latter process is outlined briefly. When adapted to dilute environments, hyperosmoregulating crustaceans counterbalance the permanent passive losses of salts along the concentration gradient and osmotic influxes of water by reductions of the permeabilities of their body surfaces to salts and water (Gross, '57; Lockwood, '77; Kirschner, '79), increased rates of urination (Oglesby, '81), and "uphill" absorption of ions across the gills (Kirschner, '79; Siebers et al., '82; Gilles and Péqueux, '86).

Active uptake of Na^+ across the epithelial cell of the crustacean gill is considered to consist of an apical amiloride-sensitive entry step represented by an Na^+/H^+ exchanger (Kirschner et al., '73; Kirschner, '79; Gilles and Péqueux, '86; Graszynski and Bigalke, '87; Siebers et al., '89) and the extrusion from the cytosol across the basolateral membrane into the hemolymph by means of the ouabain-sensitive monovalent cation pump (Kirschner, '79; Towle, '81, '84; Siebers et al., '85, '86).

Transbranchial influx of Cl^- in crabs was suggested to proceed via an apically located SITS-sensitive Cl^-/HCO_3^--exchange system (Lucu, '89; Onken and Graszynski, '89; see also Lee and Pritchard, '85) and a DPC-sensitive Cl^- channel located at the basolateral membrane (Drews and Graszynski, '87; Bianchini et al., '88; Gilles et al., '88; Onken and Graszynski, '89; Siebers et al., '90).

Several publications suggested that the counterions H^+ and HCO_3^- being exchanged against Na^+ and Cl^- by the apical electroneutral Na^+/H^+ and the Cl^-/HCO_3^- exchange systems are made available from respiratory CO_2 by the activity of the carbonic anhydrase present in dissolved form in the cytosol of the branchial cells (Fig. 6) (Burnett et al., '81; Henry and Cameron, '83; Henry, '84). The entrances of Na^+ and Cl^- from the external medium into the epithelial cells thus depend on the concerted action of the apical ion exchange systems and CA. This assumption goes back to a proposal by Krogh ('38), who argued that in aquatic animals Na^+ and Cl^- can be taken up electroneutrally and independently of each other by exchange against an equally charged ion.

Assumptions that branchial CA may play a role in osmoregulatory active ion uptake across the gills were in part based on various reports that Na-K-ATPase, which catalyzes the primary energy consuming process in active Na^+ translocation, and CA share a remarkably high number of common properties (Henry, '84). Both enzymes occur in high activities in the gills, being predominantly localized in the posterior gills of euryhaline crabs, which are considered as main ion transporting organs. Both enzymes are concentrated in the dark areas close to the afferent vessels of gill lamellae (Copeland and Fitzjarrell, '68; Neufeld et al., '80; Henry and Cameron, '82; Towle and Kays, '86). Na-K-ATPase is a marker enzyme for basolateral plasma membranes, and the bound form of CA has been shown to be present in the same region of the density gradient (see above) (Henry et al., '86; Towle and Hølleland, '87; Henry, '88a; Böttcher et al., '90a,b). Assumptions, however, that CA is partially localized in basolateral plasma membranes cannot be deduced from the results of density gradient centrifugations because in crustacean gills marker enzymes for apical and intracellular membranes

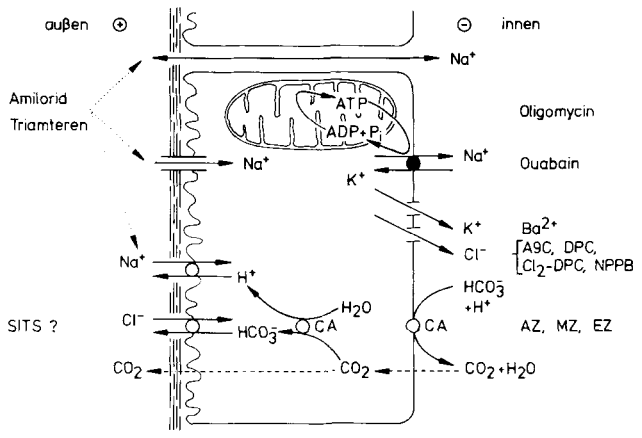

Fig. 6. *Carcinus maenas*. Model of the gill cell of the shore crab with identified and supposed ion transport proteins and their respective inhibitors. (Modified from Siebers et al., '87.)

are not yet available. At present these assumptions are exclusively based on results from experiments employing the impermeable inhibitors QAS and DBI.

Crustacean species hyperosmoregulating their body fluids when exposed to dilute media exhibit severalfold increases in activity of both branchial enzymes (Towle et al., '76; Neufeld et al., '80; Henry and Cameron, '82; Siebers et al., '82; Böttcher et al., '90b). In stenohaline species the activities of both enzymes, CA and Na-K-ATPase, are distributed homogenously over anterior and posterior gill pairs (Henry and Cameron, '82; Wheatly and Henry, '87).

Consistent with these ideas is the proposal by Burnett et al. ('81) that crab gill CA plays a major role in Cl^- ion regulation. Acetazolamide (2×10^{-4} M) added to the seawater significantly increased hemolymph Cl^- concentrations in crabs *Pachygrapsus crassipes* maintained in dilute seawater. In crabs acclimated to normal seawater and higher salinities, hemolymph Cl^- levels did not change.

In crabs *Callinectes sapidus* acclimated to brackish water of 250 mOsm, inhibition of carbonic anhydrase by injections of AZ to establish a hemolymph concentration of 10^{-4} and 10^{-3} M did reduce the animals' ability to hyperregulate hemolymph osmolality and concentrations of Na^+ and Cl^- (Henry, '84). These results were confirmed by the same author (Henry, '87b). In crabs *C. sapidus* injections of acetazolamide and benzolamide to obtain a final hemolymph concentration of 10 μM resulted in a rapid decrease in hemolymph osmolality and Na^+ and Cl^- concentrations. In addition, these drugs induced a respiratory acidosis (increase in the partial pressure and total concentration of CO_2 and decrease in pH), an effect also observed in crabs treated with the membrane-impermeable CA inhibitor QAS (see preceding paragraph). Based on his findings that QAS had no effects on hemolymph osmolality and ion concentrations, the author developed his concept of two distinct CA localizations and functions within the branchial cell. According to this concept, the enzyme associated with the basal membrane is inhibitable by QAS, acetazolamide, and benzolamide. It functions in the dehydration of hemolymph bicarbonate, whereas the CA dissolved in the cytosol is accessible only to the permeable drugs, acetazolamide and benzolamide, but not to QAS. The cytosolic enzyme was considered to catalyze the conversion of CO_2 penetrating into the cell and the CO_2 generated through cellular metabolism to HCO_3^- and H^+, which are then exchanged against ambient Na^+ and Cl^- through the apical ion exchange systems (Fig. 6). The compartmentation of CA within the branchial cell is therefore assumed as prerequisite to fulfill both functions, CO_2 excretion and ion uptake, in a single organ (Henry, '88b).

These concepts did not remain without contradictions. Doubts about an osmoregulatory ion transport function of branchial CA were reported by various authors. It was shown by Burnett and Towle ('90) that in isolated perfused gills of the crab *Callinectes sapidus* acclimated to 5 ‰ S, a near-physiological gradient of HCO_3^- of 5 mM:1.4 mM between internal perfusate and external bath did not drive a portion of unidirectional influx of Na^+ from the bath to the perfusate (representing the hemolymph space). Influx of Na^+ proceeding at a maximum rate of $3.2\ \mu mol \times g^{-1} \times min^{-1}$ against a large Na^+ concentration difference of 25 mM (bath) :273 mM (perfusate) was reduced to ca. 25% of control values by addition of 10 mM sodium cyanide or sodium iodoacetate. Additional application of 1 mM internal (perfusate) AZ resulted in a very small decrease in Na^+ influx, which was attributable to a parallel increase of branchial resistance measured as a small increase in the pressure driving the perfusate through the gill. A comparable effect was observed using 10^{-3} M internal ethoxzolamide; in this case the internal perfusate was free of any form of CO_2. The perfusion experiments performed in the presence of AZ indicate that passive movement of inorganic carbon through the gill from hemolymph to external medium is not responsible for the sodium influx remaining after CN^- poisoning of the gill.

Using isolated perfused gills of shore crabs *C. maenas*, Böttcher et al. ('91) tested whether CA inhibitors affected transepithelial potential differences and transbranchial unidirectional influxes of radiolabeled Na^+ and Cl^-. First the gills were bathed and perfused with 50% seawater in a symmetrical mode (Fig. 7), then a gradient of 6 mM $NaHCO_3$ was applied. In this case the symmetry of the incubation media was maintained by addition of the respective amounts of Na^+-gluconate to the bath. The pH was adjusted to 7.75 with 0.5 mM Tris and varying concentrations of HEPES. In no case were the potential differences and unidirectional influxes of Na^+ (data not shown) and Cl^- (Fig. 7) of controls changed significantly when 10^{-4} M acetazolamide was added to the bath and to the perfusion solution (Böttcher et al., '91). Changing the incubation media from symmetrically applied 50% seawater (ca. 1 mM HCO_3^-) to a near-physiological bicarbonate gradient of 6 mM directed from perfusate to bath had no influence on unidirectional

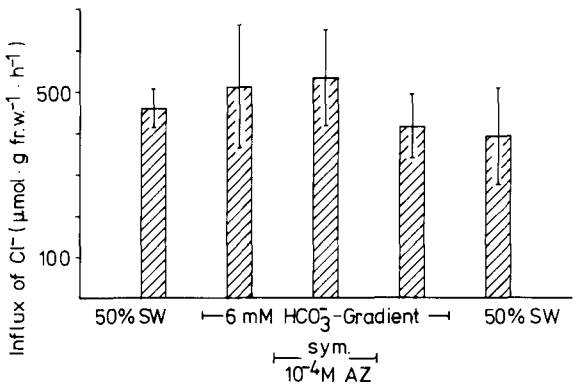

Fig. 7. *Carcinus maenas*. Influx rates of chloride ions across isolated perfused posterior gills in relation to bicarbonate gradients (0 and 6 mM) directed from the perfusion to the bathing solution, and in the presence of symmetrically applied 10^{-4} M acetazolamide (AZ). Data represent means ± standard deviations obtained from a sample size of 3 gills. SW = seawater. (From Böttcher et al., '91.)

influxes of Na^+ (data not shown) and Cl^- (Fig. 7). In contrast to the missing effects of an HCO_3^- gradient and AZ application on PDs and ion transfer rates, it was reported in the preceding paragraph that under the same conditions the unidirectional transport of inorganic carbon depended on the magnitude of the gradient and was greatly susceptible to the presence of AZ.

To date, the presence of an apically located electroneutral Cl^-/HCO_3^- exchange protein has been demonstrated only indirectly. The question of which proportion of Cl^- is taken up by the (hypothetical) Cl^-/HCO_3^- exchanger can be solved experimentally. Following Cameron ('78), unidirectional Cl^- influxes in intact crabs *Callinectes sapidus* are high in individuals acclimated to fresh water, approximately three times as high as CO_2 effluxes calculated from Aldridge and Cameron ('79). A CO_2 excretion rate of 3.2 µmol × g^{-1} × h^{-1} at room temperature thus corresponded to a Cl^- influx of 9.6 µmol × g^{-1} × h^{-1} (Table 2). These results were confirmed using intact crabs *Carcinus maenas* acclimated to 10 ‰ brackish water. Uptake of Cl^- in these crabs was 7.6 ± 1.9 (n = 4) µmol × g^{-1} × h^{-1} (Table 2; Siebers et al., unpubl.). Excretion rates of total CO_2 calculated from oxygen uptake (Siebers et al., '82) using the conversion factor published by Aldridge and Cameron ('79) amounted to 2.2 ± 0.6 (n = 8) µmol × g^{-1} × h^{-1} (Table 2). These calculations were based on the assumption that the CO_2 excretion (estimated from O_2 uptake) is a net efflux which equals the unidirectional efflux of CO_2. Unidirectional chloride uptake and CO_2 excretion rates in isolated perfused gills differed comparably (Table 2).

TABLE 2. Uptake of Cl^- and excretion of total CO_2 in intact crabs and isolated perfused gills[1]

Species	Cl^- uptake	CO_2 excretion
Callinectes sapidus[2] (acclimated to fresh water)	9.6[4]	3.2[5]
Carcinus maenas (acclimated to 10 ‰ S)	7.6[6]	2.2[7]
Carcinus maenas[3]	280–460[8]	99[9]

[1]Gills were perfused and bathed with 50% seawater; the HCO_3^- gradient between hemolymph space and external bath was 6 mM. Data are presented in terms of µmol · g fresh weight^{-1} · h^{-1}.
[2]Intact crabs.
[3]Isolated perfused gills.
[4]Cameron ('78).
[5]Aldridge and Cameron ('79).
[6]Siebers et al. (unpubl.).
[7]Siebers et al. ('72).
[8]Lucu and Siebers ('87); Siebers et al. ('90); Böttcher et al. ('91).
[9]Böttcher et al. ('91).

The comparison of Cl^- uptake and CO_2 excretion rates in intact crabs and perfused gills suggests that even under the assumption that CO_2 is excreted quantitatively via the apical exchange system, only about 30% of total Cl^- influx is covered by HCO_3^- efflux. This percentage is certainly an unrealistically high estimation since only a portion of total CO_2 excretion can be assumed to be liberated as bicarbonate, the rest diffusing as CO_2.

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