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Carbonic anhydrase, a respiratory enzyme in the gills of the shore crab *Carcinus maenas*

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ABSTRACT: This paper summarizes investigations on the enzyme carbonic anhydrase (CA) in the gills of the osmoregulating shore crab *Carcinus maenas*. Carbonic anhydrase, an enzyme catalyzing the reversible hydration of CO_2 to HCO_3^- and H^+, is localized with highest activities in the posterior salt-transporting gills of the shore crab – and here CA activity is strongly dependent on salinity. Contrary to the earlier hypothesis established for the blue crab *Callinectes sapidus* that cytoplasmic branchial CA provides the counter ions HCO_3^- and H^+ for apical exchange against Na^+ and Cl^-, the involvement of CA in NaCl uptake mechanisms can be excluded in *Carcinus*. Differential and density gradient centrifugations indicate that branchial CA is a predominantly membrane-associated protein. Branchial CA was greatly inhibited by the sulfonamide acetazolamide (AZ) K_i = $2.4 \cdot 10^{-8}$ mol/l). Using the preparation of the isolated perfused gill, application of 10^{-4} mol/l AZ resulted in an 80 % decrease of CO_2/HCO_3^- excretion. Thus we conclude that CA is localized in plasma membranes, maintaining the CO_2 gradient by accelerating adjustment of the pH-dependent CO_2/HCO_3^- equilibrium.

INTRODUCTION

The enzyme carbonic anhydrase (CA) catalyzes the reversible hydration of CO_2 and H_2O to HCO_3^- and H^+. The presence of CA has been described in many tissues among the vertebrates and to a smaller extent in invertebrates. CA is involved in several physiological processes, e.g. respiration, ion transport, acid-base-regulation, and calcification (for reviews see Maren, 1967; Henry, 1988b; Gros et al., 1988).

Considerably high activities of CA were found in the gills of euryhaline crustaceans (Henry & Cameron, 1982a, b) which are known to regulate their hemolymph osmolality when exposed to low salinities, in order to compensate for passive ion loss. In such euryhaline species CA activity is strongly dependent on salinity. It was increased in gills of crabs acclimated to diluted media (Henry & Cameron, 1982a; Wheatly & Henry, 1987). Therefore the enzyme was supposed to support those gill functions necessary for life in reduced salinity, and it was concluded that gill CA could be directly involved in ion transport processes. In detail, gill CA was described as a preponderantly cytosolic enzyme, providing apical Na^+/H^+- and Cl^-/HCO_3^--exchangers with HCO_3^- and H^+ by

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means of hydrating metabolic CO_2 (Henry & Cameron, 1982a; Henry, 1984; Burnett et al., 1985). Only a very small amount of CA was considered to be localized in association with the basolateral membranes facilitating CO_2 excretion by dehydrating hemolymph HCO_3^- (Henry, 1987, 1988a).

In the following, a short overview on CA in the gills of the euryhaline shore crab *Carcinus maenas* will be presented – dealing with salinity-dependence, subcellular localization and physiological role of the enzyme.

MATERIAL AND METHODS

Shore crabs *Carcinus maenas* were obtained from fishermen in Kiel Bay (Baltic Sea). In the laboratory, the crabs were held in short daylight periods of 6 h in order to avoid moulting. The temperature was 18 °C. The crabs were fed three times a week with bovine heart, and were starved 24 h prior to experimental use. Acclimation to different salinities lasted for at least 4 weeks.

Activity of CA was determined according to Maren (1960), and modified by Bruns et al. (1986), measuring the duration of the development of HCO_3^- and H^+ from CO_2 using phenol red as an indicator. The activity A was defined according to the equation

$$A = t_0/t_{enz} - 1,$$

where t_0 means the duration without enzyme and t_{enz} is the duration of the reaction supported by catalytic activity of CA. One unit (U) of CA activity was defined as the concentration of enzyme in the final assay volume halving the uncatalyzed reaction time (Maren et al., 1954). In order to record total CA activity, 1 % Triton X-100 was added in the assay.

CA was isolated according to Whitney (1974). To determine the inhibition kinetics of the isolated enzyme, various concentrations of its specific inhibitor acetazolamide (AZ) were applied in the assay. The inhibition constant K_i was calculated by means of the Easson and Stedman plot (1937).

Posterior gills were perfused according to Siebers et al. (1985), with a flow rate of 0.13 ml·min^{-1}. During perfusion, transepithelial potential differences (PD) were monitored using a millivolt metre (type 187, Keithley), connected with the perfusate and the bath solutions by means of two Ag/AgCl electrodes (type 373-S7, Ingold, Frankfurt/M.). Perfusion and bath solutions were 50 % seawater (200 mmol/l Na^+), buffered by the addition of 0.5 mmol/l Tris (Sigma). In order to establish a HCO_3^- gradient between the hemolymph side and the external medium, 6 mmol/l $NaHCO_3$ was added to the perfusate. For compensation of the Na^+ added in the perfusate, the bath solution was enriched by 6 mmol/l Na-gluconate. Finally the solutions were adjusted to pH 7.8 by means of HEPES. The CA inhibitor AZ was added prior to final pH adjustment. The tracer was applied as $NaH^{14}CO_3$ (Amersham) at the specific activity of 10 µCi per 10-ml perfusate. Undirectional effluxes of $H^{14}CO_3^-$ from perfusate to bath solution were measured in 2-ml aliquots taken at five 5-min intervals from the bath. Influxes of Na^+ and Cl^- from bath to internal perfusate were determined in separate experiments, while perfusate was collected after passage through the gill at four 5-min intervals. ^{22}Na and ^{36}Cl (Amersham) were applied as NaCl at specific activities of 12 µCi ^{22}Na and 5.6 µCi ^{36}Cl per 40-ml bath solution. ^{14}C and ^{36}Cl radioactivities were detected in a liquid scintillation counter (Prias,

Packard), ^{22}Na was quantified using a gamma-counter (Fischer, Hamburg). Fluxes were expressed in terms of $\mu mol \cdot g^{-1}$ fresh wt.$\cdot h^{-1}$.

In order to examine subcellular distribution of branchial CA, gills from crabs acclimated to a salinity of 10‰ were homogenized (1:20) with buffer (250 mmol/l sucrose, 10 mmol/l Tris-HCl, 0.2 mmol/l EDTA, pH 7.5) and then centrifuged at 100000 g for 1 h at 4 °C (ultracentrifuge L8-70M, Beckman, rotor 70.1. Ti.). After decanting supernatants (SI), pellets (PI) were resuspended in homogenizing buffer, homogenized again and sonicated twice for 5 sec (sonicator type GTS 22/125, sonotrode Tu 157/4, KLN Ultraschall, Heppenheim). The pellets were recentrifuged. The procedure was repeated twice, all in all resulting in SI, PI, SII, PII, SIII, and PIII.

For a more specific localization of CA, density gradient centrifugation was employed according to Towle & Hølleland (1987) and Henry (1988a). Homogenates (1:10) of posterior gills were centrifuged at 750 g for 15 min at 4 °C. After washing the resulting pellet twice, the combined 750 g supernatants (SI) were centrifuged at 7500 g (20 min, 4 °C). The resulting pellet contained membranes and mitochondria and was layered on the top of a 33.5 ml continuous sucrose gradient (10–40 %, w/w). Following centrifugation of the gradient at 100000 g for 1.5 h (rotor SW27, Beckman), 30 fractions of approx. 1.3 ml were isolated from the gradient. CA activity, protein concentration (BCA, Pierce) and the marker enzymes Na-K-ATPase (Siebers et al., 1982), lactate dehydrogenase (Bergmeyer, 1974), and cytochrome oxidase (Wharton & Tzagoloff, 1967) were measured.

RESULTS

In order to analyse salinity dependence of CA activity in the branchial tissue of *Carcinus maenas,* crabs were acclimated to different salinities (10, 20, 30, 40, and 50‰). As shown in Figure 1, anterior gills (1–6) comprised CA activities around 10 U$\cdot$mg protein^{-1} and these activities were not influenced by the acclimatization salinity. In the posterior gills (7–9), CA activities were found to be higher, and they obviously increased with decreasing salinities. The greatest increase of CA activity up to 40 U$\cdot$mg protein^{-1} was observed in posterior gills from crabs acclimated to the lowest salinity of 10‰.

The physiological function of the enzyme was investigated by means of the preparation of the isolated, perfused gill. Using a nearly-natural bicarbonate gradient of 6 mmol/l directed from internal perfusate to external bath, the HCO_3^--fluxes were about 100 $\mu mol \cdot g$ fr.wt.$^{-1} \cdot h^{-1}$ (Fig. 2). External application of 10^{-4} mol/l AZ resulted in a drop in efflux rate of 80 %. Different concentrations of internal AZ (10^{-4}, $5 \cdot 10^{-4}$, 10^{-3} mol/l) reduced bicarbonate efflux to a corresponding extent. Recovery of efflux rates after removing the inhibitor amounted to ca 60 % of the initial efflux rate. In no instance was PD affected by AZ. Under symmetrical bicarbonate conditions (50 % seawater, including about 1 mmol/l HCO_3^-), influxes of Na^+ (1120 ± 143 $\mu mol \cdot g^{-1}$ fr.wt.$\cdot h^{-1}$, $n = 4$) and Cl^- (460 ± 47 $\mu mol \cdot g^{-1}$ fr.wt.$\cdot h^{-1}$, $n = 3$) exceeded HCO_3^- effluxes by severalfold (data not shown). Neither establishment of the 6 mmol/l HCO_3^- gradient, nor symmetrically applied AZ (10^{-4} mol/l) resulted in significant deviations from control fluxes.

Distribution of CA activity within the posterior gills of the shore crab was determined by analysing activities of CA and LDH, a cytoplasmic marker enzyme, after repeated centrifugations of homogenates and the resulting pellets. In order to secure quantitative release of soluble CA into the supernatants, pellets were rehomogenized and sonicated

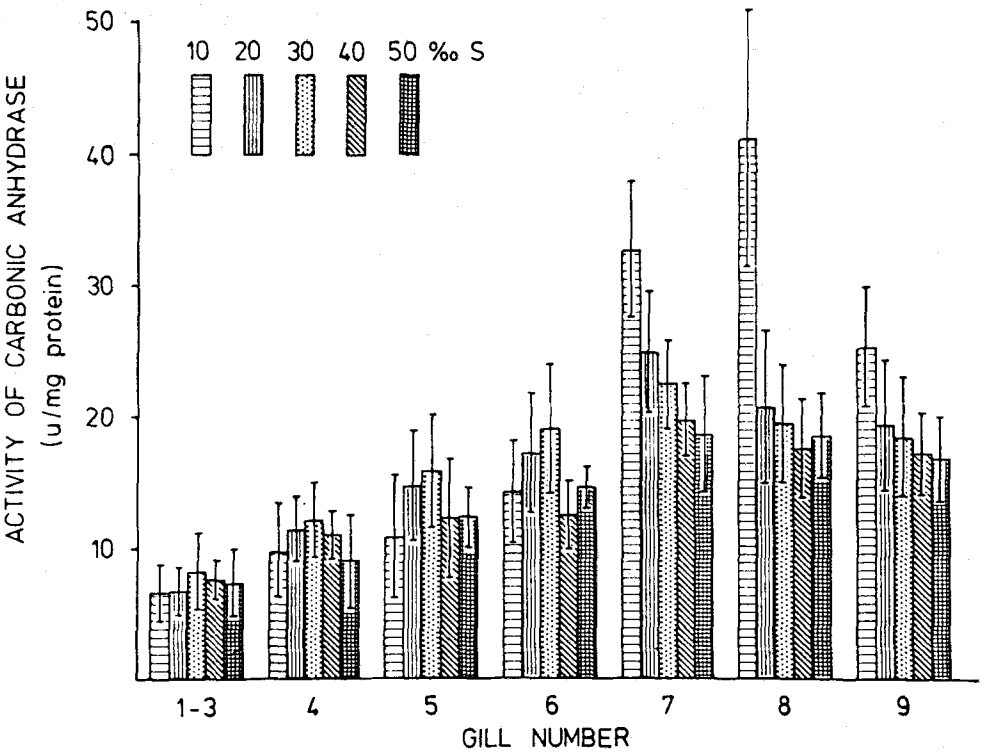

Fig. 1. Carbonic anhydrase activities in the gills of the shore crab *Carcinus maenas* acclimated to various salinities. Data represent means ± SD (n = 6). (Figure taken from Böttcher et al. [1990a], by permission of Wiley, New York, NY 10158-0012).

prior to recentrifugation. Following these procedures, we found that about 90 % of CA activity was bound (PI). Repeated centrifugation and sonification did not result in liberation of more than 10 % of CA into the supernatants. LDH activity was mainly present in SI (82 %). Only a small amount of the enzyme (3 %) was released by repeated centrifugation and sonification of the pellets. For further localization of the bound CA activity, density gradient centrifugation was applied. A 7500 g-pellet including mitochondria, membranes and other particular cell components was fractionated in a 10–40 % sucrose gradient. CA activity paralleled activity of Na-K-ATPase, a marker enzyme of plasma membranes (Fig. 3). Fractions comprising mitochondria as indicated by the presence of cytochrome oxidase activities were free of CA. Lack of LDH activity ensured separation of soluble cell components by differential centrifugation prior to density gradient centrifugation.

Figure 4 shows the effect of the specific sulfonamide inhibitor AZ on the activity of isolated branchial CA. A concentration of 10^{-6} mol/l was sufficient to inhibit CA activity totally. The inhibition constant K_i of $2.4 \cdot 10^{-8}$ mol/l was calculated according to Easson & Stedman (1937).

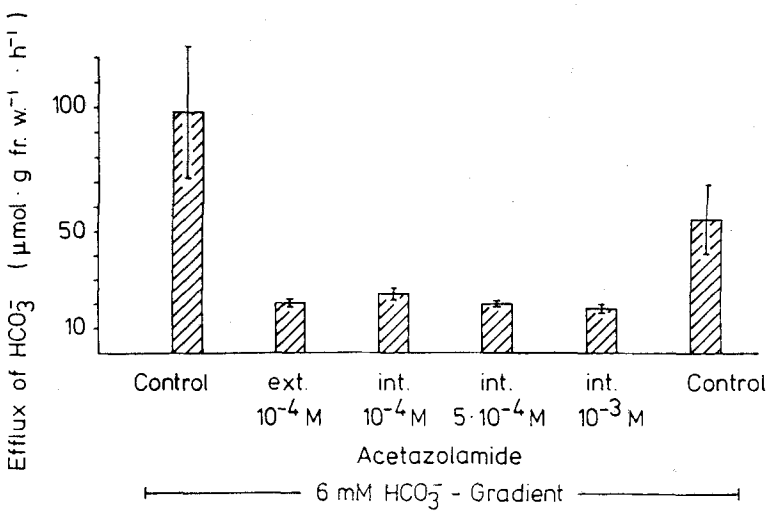

Fig. 2. Efflux of bicarbonate across posterior gills of the shore crab *Carcinus maenas* acclimated to 10 ‰ S. Perfusate contained 6 mmol/l HCO_3^-. The specific inhibitor acetazolamide was applied in the bath (10^{-4} mol/l) and in the perfusate (10^{-4}, $5 \cdot 10^{-4}$, 10^{-3} mol/l). Data represent means ± SD (n = 4). (Figure taken from Böttcher et al. [1991], by permission of Springer, Heidelberg, Germany)

DISCUSSION

Inhibition of isolated branchial CA by AZ revealed a high affinity of crab CA to this specific sulfonamide CA inhibitor. The K_i of $2.4 \cdot 10^{-8}$ mol/l was similar to previously determined inhibition constants using crude homogenates (Böttcher et al., 1990a). In this high affinity to sulfonamide inhibitors, crab CA resembles mammalian isozymes, i.e. cytosolic CA II and CA IV, the membrane-bound form. Using AZ, Henry (1988a) found a K_i of $7.8 \cdot 10^{-9}$ mol/l for cytoplasmic CA and K_i of $6.7 \cdot 10^{-9}$ mol/l for the membrane-bound gill CA of *Callinectes sapidus*.

High activities of CA in the gills are typical of all crustaceans so far investigated. Increased specific activities in posterior gills were found in the semiterrestrial crab *Cardisoma guanhumi*, the terrestrial crab *Gecarcinus lateralis* (Henry & Cameron, 1982a) and the euryhaline crabs *C. sapidus* (Henry & Cameron, 1982a), *Eriocheir sinensis* (Olsowski et al., 1995; this volume), and *Carcinus maenas*. CA activity was salinity-dependent in *C. sapidus*, *E. sinensis*, *C. maenas*, and in the freshwater crayfish *Pacifastacus leniusculus* (Wheatly & Henry, 1987). Increased activities in posterior gills and salinity-dependence have been also described for the Na-K-ATPase (Towle, 1981, 1984; Siebers et al., 1982; Towle & Kays, 1986), an enzyme functioning in active ion transport across gill epithelium. These corresponding properties of Na-K-ATPase and CA led to the assumption that CA could also support ion transport by providing apical ion-exchangers Na^+/H^+ and Cl^-/HCO_3^- with H^+ and HCO_3^-. However, considering present physiological data, the assumed direct involvement of CA in ion transport processes must be considered questionable.

Since PDs were shown to be correlated with active Na^+ transport across the gill epithelium (Siebers et al., 1986), unchanged PDs during altering HCO_3^- conditions or

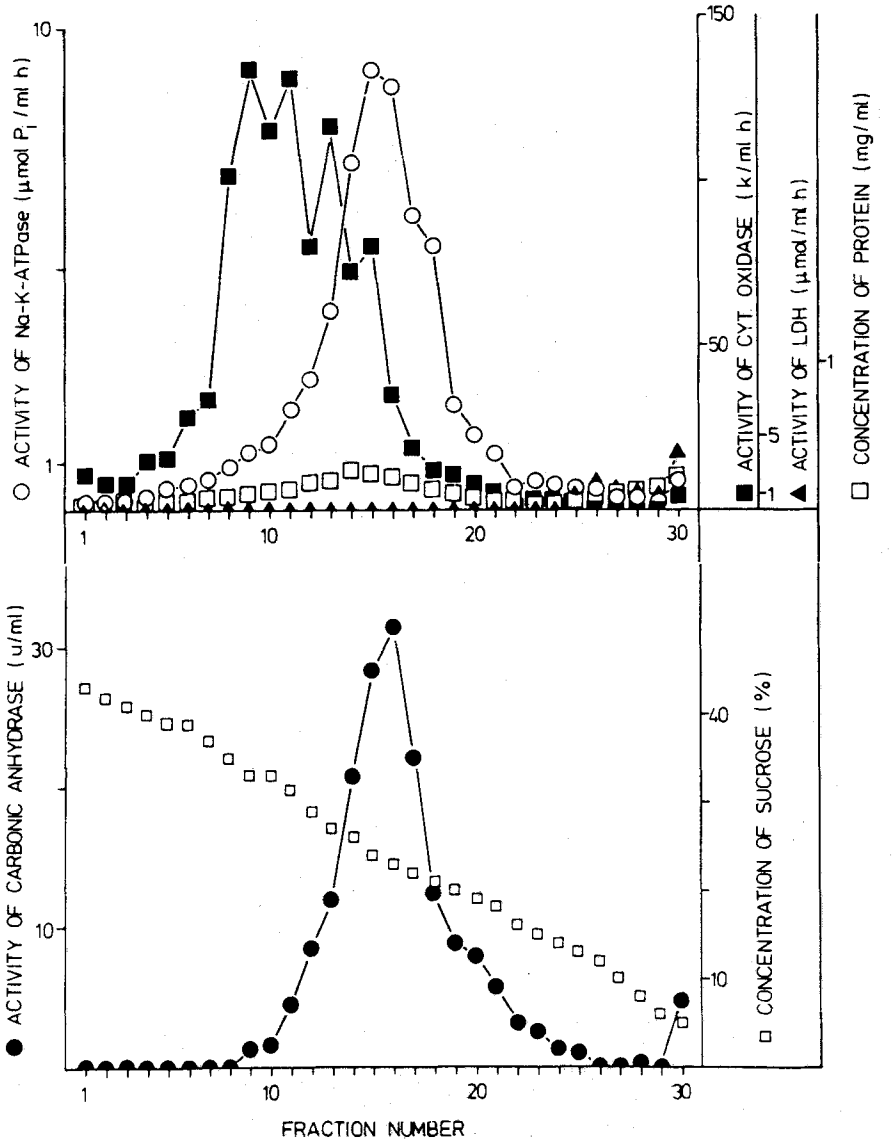

Fig. 3. Activities of carbonic anhydrase, Na-K-ATPase, and cytochrome oxidase in the fractions obtained from density gradient centrifugation. Homogenates of posterior gills from shore crabs acclimated to 10‰ S were centrifuged at 750 g. The resulting pellet was rehomogenized and centrifuged again at 7500 g. The resuspended 7500 g pellet was layered on the top of a 10–40 % (w/w) sucrose gradient and was centrifuged at 100000 g. (Figure taken from Böttcher et al. [1990b], by permission of Pergamon Press, Headington Hill Hall, Oxford OX3 OBW, UK)

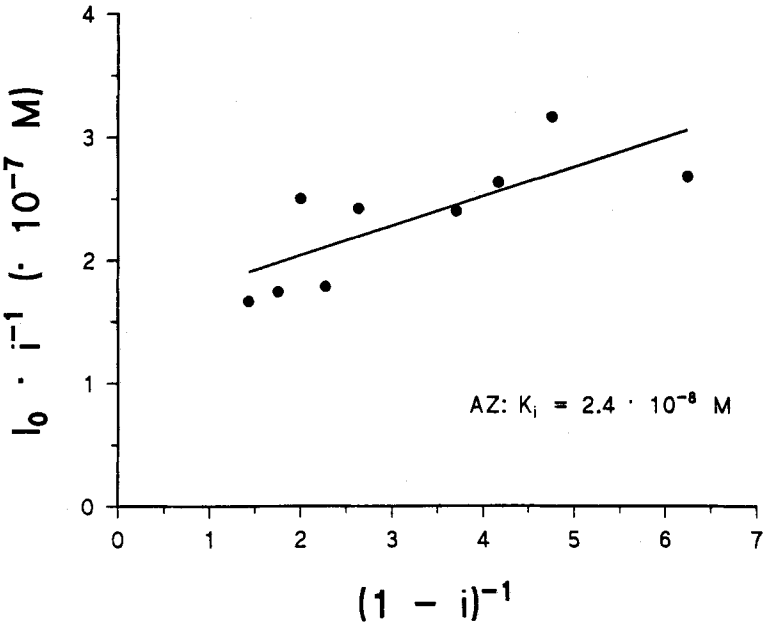

Fig. 4. Inhibition kinetics of branchial carbonic anhydrase. Concentrations between 10^{-5} and 10^{-10} mol/l acetazolamide were applied in the test. K_i was determined by means of the Easson & Stedman plot (1937) based on the equation: $I_0/i = K_i/(1-i) + E_0$, where E_0 represents the total concentration of enzyme, K_i the inhibition constant, and i the fractional inhibition of enzyme activity at an inhibitor concentration of I_0

inhibition of CA clearly exclude participation of CA in Na^+ transport. These results were recently confirmed by Onken & Siebers (1992), using single split lamella preparations of posterior gills of shore crabs acclimated to low salinity. Internal AZ (10^{-4} mol/l) did not change short-circuit currents and resistances of the epithelium. Injection of 10 and 15 mg AZ/100 ml hemolymph into the blue crab *C. sapidus* also did not affect transepithelial potentials between the body fluids of the crab and the external medium, as shown by Cameron (1979).

Controversial results were published by Onken & Graszynski (1989). After perfusion of posterior gills of *E. sinensis* with Na^+ free solutions and 10^{-3} mol/l AZ, the authors found significantly reduced PDs. Perhaps such high concentrations of AZ may cause unspecific effects on other transport proteins. Anyway, we have to consider species-dependent differences in epithelial transport capacities, since chinese crabs tolerate freshwater and shore crabs only migrate into brackish water.

Ehrenfeld (1974) demonstrated that intraperitoneally injected $2 \cdot 10^{-4}$ mol/l AZ inhibited net fluxes of Na^+ and Cl^- in the crayfish *Astacus leptodactylus*. Hemolymph osmolalities and ion concentrations in *C. sapidus* were reduced after injection of 10^{-4} and 10^{-3} mol/l AZ (Henry & Cameron, 1963; Henry, 1987). The data obtained by measuring ion fluxes across the gills of *C. maenas* are in contrast to the findings mentioned. Comparison of the influx rates of Na^+ (1120 ± 143 $\mu mol \cdot g^{-1}$ fresh wt.$\cdot h^{-1}$) and Cl^- (460 ± 47 $\mu mol \cdot g^{-1}$ fresh wt.$\cdot h^{-1}$) with the efflux rates of HCO_3^- (99 ± 27 $\mu mol \cdot g^{-1}$ fresh

wt.·h^{-1}) and the unchanged NaCl influxes following inhibition of CA reaffirm the minor role of Na^+/H^+ and Cl^-/HCO_3^- exchange in ion regulation of NaCl, and the involvement of branchial CA in such processes.

On the other hand, we could clearly demonstrate the role of branchial CA in CO_2 excretion. In order to simulate natural conditions, a 6 mmol/l HCO_3^- gradient was applied between perfusate and bath. At a pH of 7.8, 95 % of total CO_2 are present as HCO_3^-, and only 5 % as CO_2 (Cameron, 1979). Inhibition of CA by either external or internal AZ led to an 80 % reduction of HCO_3^- efflux, indicating that 80 % of CO_2 excretion is dependent on CA activity. Following Burnett et al. (1985), part of branchial CA is localized in the basolateral membranes, dehydrating hemolymph HCO_3^- to CO_2 in order to maintain the CO_2 gradient by accelerating adjustment of the pH-dependent HCO_3^-/CO_2 equilibrium.

Localization of CA in the gills of *C. maenas* by means of differential and density gradient centrifugations, resulted in the assumption that the bulk of branchial CA (90 %) is associated with plasma membranes; the remaining 10 % CA found in the supernatants may have been released by rough sample treatment. These data are in contrast to the results obtained by Henry (1988a), who found 93.5 % of the total branchial CA concentration of *C. sapidus* in a soluble form and only the smaller amount of 6.5 % located in plasma membranes. According to Olsowski et al. (1995, this volume), approximately 80 % of the whole branchial CA activity of *E. sinensis* is bound to membranes.

Since a suitable marker enzyme for apical plasma membranes is not yet available and AZ is known to easily permeate lipid membranes, we cannot decide whether branchial CA of *C. maenas* is localized apically, basolaterally, or lining the whole cell surface.

Acknowledgement. This work is a summary of data dealing with branchial CA of the shore crab, and is based on previous publications by Böttcher et al. (1990a, b; 1991).

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