Biochimica et Biophysica Acta, 689 (1982) 143–154
Elsevier Biomedical Press

BBA 71234

SALINITY ADAPTATION OF HCO_3^--DEPENDENT ATPase ACTIVITY IN THE GILLS OF BLUE CRAB *(CALLINECTES SAPIDUS)*

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(Received February 15th, 1982)

Key words: -HCO_3^--ATPase; Salinity adaptation; (Crab gill microsome)

A bicarbonate-dependent ATPase (EC 3.6.1.3) was found in microsomal preparations from blue crab gills. When the crabs were transferred to low salinity (200 mosmolal) from seawater (1000 mosmolal), the HCO_3^--dependent ATPase increased in all gill pairs, reaching its new steady state in 2 weeks. The greatest increase occurred in the sixth and seventh gill pairs (approx. 2.5-fold). Maximal enzyme activity was observed at an Mg^{2+} concentration of 3 mM and an optimal pH of 7.8. The apparent K_a for HCO_3^- was found to be 8.9 mM. Kinetic analysis showed that low-salinity adaptation increased the V_{max} without altering the K_m for ATP. When the microsomes from high-salinity crab gills were treated with detergent or assayed at different temperatures, the total enzyme activity did not reach the activity levels seen after adaptation to low salinity. These results suggest that the alteration of HCO_3^--ATPase activity may be due to synthesis, rather than modulation of membranes or of the existing enzyme activity.

Introduction

A bicarbonate-dependent, thiocyanate-inhibited ATPase (ATP phosphohydrolase, EC 3.6.1.3) was first described in frog gastric mucosa by Kasbeker and Durbin [1], where it was thought to play a role in acid secretion and the associated reabsorptive transport of HCO_3^- [2,3]. A similar enzyme has also been described in mammalian pancreas [4], intestinal and renal brush border membranes [5–8], and in the gills of *Necturus* [9], rainbow trout [10] and eel [11], all sites of HCO_3^- (Cl^-) transport. Thus, a role for this enzyme has been proposed in ion transport [3]. Recently, however, the participation of this enzyme in active transport of ions has been questioned because of uncertainty concerning the intracellular localization of the enzyme. Van Amelsvoort et al. [12] concluded that HCO_3^--ATPase originates exclusively from mitochondria in rainbow trout gill, rabbit kidney and rabbit gastric mucosa. A mitochondrial localization of this enzyme has also been reported in gastric mucosa and intestinal epithelium of the rat [13,14] and gill tissue of Japanese eel [15]. However, Kerstetter and Kirschner [10] demonstrated the presence of this enzyme in both mitochondrial and microsomal fractions from the gills of rainbow trout and Bornancin et al. [16] have recently confirmed the microsomal localization of anion-dependent ATPase in gills of the rainbow trout and concluded that this enzyme is involved in gill anion exchanges related to mineral and acid-based regulation in the gills of goldfish and eel [17,18]. De Pew and Towle [19] also found substantial HCO_3^--stimulated ATPase activity in purified plasma membrane fractions as well as in

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PCMBS, *p*-chloromercuribenzenesulfonic acid.

mitochondrial fractions of fiddler crab. The existence of HCO_3^--stimulated ATPase in the absence of mitochondrial contamination has been also demonstrated in brush border membranes of the rabbit renal tubule by Liang and Sacktor [8] and the rat kidney cortex by Kinne-Saffran and Kinne [7].

We have begun to study the functional role of this enzyme in salinity adaptation and ion transport across the gill of a euryhaline decapod crustacean, the blue crab (*Callinectes sapidus*). The gill is an important site of osmotic, ionic and acid-base regulation in crustaceans and the membrane-bound ATPases have been implicated in the movement of ions across the gill epithelium [16–26]. The most important osmotically active solutes in this system are Na^+ and Cl^-. When a hyperosmotic osmoregulator, like the blue crab, is exposed to a dilute environment, it increases its electrolyte uptake with an associated increase in gill $(Na^+ + K^+)$-ATPase activity [27–32]. However, despite the demonstration of Cl^--HCO_3 exchange and of HCO_3^--activated ATPase in gills (and intestine) of some marine organisms [16–22], its role in adaptation to osmotic stress has not been thoroughly investigated. The results presented in this communication show that low-salinity adaptation of the blue crab results in increased HCO_3^--dependent, SCN^--sensitive ATPase activity of a gill microsomal preparation, and describes the physicochemical properties of this enzyme.

Materials and Methods

Materials. Sodium bicarbonate and vanadate were purchased from Fisher Scientific Co. and sodium thiocyanate from Mallinckrodt, Inc. Triton X-100, oligomycin, cytochrome *c*, acetazolamide, ouabain, *p*-chloromercuribenzenesulfonic acid, dithiothreitol and all the nucleotides were obtained from Sigma Chemical Co. All other chemicals were of reagent grade purity.

Experimental animals. Blue crabs (*C. sapidus*), 170–230 g body weight, were obtained from commercial fishermen in the area of St. Augustine, FL. The animals were kept in aerated seawater (1000 mosmolal) at 13–17°C for 2 weeks before experimental use. They were fed fish twice a week.

After acclimation to seawater in the laboratory, animals to be adapted to low salinity were transferred to a 250 gallon recirculating aquarium (Aquarium Systems) containing 20% seawater (200 mosmolal). The water was constantly aerated and filtered, and maintained at 16°C. Animals were killed at intervals to determine the time course of adaptation. Complete adaptation required 2 weeks exposure to the low-salinity environment (see Results).

Preparation of enzymes. The gills were excised and rinsed in ice-cold saline (0.9% NaCl). After blotting with filter paper to remove saline and hemolymph, the tissue was minced with scissors and suspended in homogenizing buffer (250 mM sucrose, 20 mM Tris-Hepes, 0.5 mM EDTA, pH 8.3) at 1 g tissue/10 ml buffer. The suspension was homogenized using a Teflon-glass Potter-Elvehjem homogenizer (20 strokes at 1000 rpm). The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at $800 \times g$ for 10 min. The pellet (nuclear fraction, N) was discarded and the supernatant was centrifuged at $10000 \times g$ for 10 min to remove the mitochondria (P_1). The supernatant was then centrifuged at $100000 \times g$ for 60 min. The resulting microsomal pellet was suspended in the same buffer at a concentration of 5 mg protein/ml (P_2). The supernatant (S) was retained for analysis.

Enzyme assay. HCO_3^--ATPase activity was measured in a reaction mixture containing 50 mM $NaHCO_3$, 3 mM $MgCl_2$, 50 mM Tris-Hepes buffer, pH 7.8, and 0.05 ml of enzyme solution (0.25–0.5 mg protein). Characterization of the enzyme properties (see Results) indicated that these conditions yielded optimal enzyme activity. After 10 min preincubation at 30°C, the reaction was started by adding 5 mM ATP. The final volume was 1.0 ml. After 15 min (the period of linear activity), the reaction was terminated by the addition of 1.0 ml of 10% trichloroacetic acid solution. Released P_i was estimated from the protein-depleted supernatant by the method of Fiske and SubbaRow [33]. Cytochrome *c* oxidase was measured according to the method described by Cooperstein and Lazarow [34]. 5′-Nucleotidase was measured by the method of Aronson and Touster [35]. Protein concentration was determined by the method of Lowry et al. [36] or a modification of the Biuret

method [37] with bovine serum albumin as standard.

Results

Microsomal HCO_3^--ATPase preparation

As shown in Table I, HCO_3^--ATPase was found in both mitochondrial and microsomal fractions, P_1 and P_2, respectively. Since the existence of this enzyme in microsomal fractions has been questioned by a number of authors, it was necessary to determine the degree of purity of the microsomal fraction. For this reason, cytochrome *c* oxidase activity, a marker enzyme for mitochondria, and 5'-nucleotidase, a marker enzyme for plasma membrane, were measured in the mitochondrial and microsomal preparations. Although the total ATPase activity was higher in the mitochondrial fraction, the cytochrome *c* oxidase activity occurred predominantly in fraction P_1 (60–80%) and had only trace activity (3%) in fraction P_2 (Table II). The specific activity of this enzyme in fraction P_2 appeared to be approx. one-twentieth of that in fraction P_1, and indicated that mitochondrial contamination in the microsomal preparation (P_2) was very small (Table II). On the other hand, the specific activity of the plasma membrane marker, 5'-nucleotidase, was significantly higher in fraction P_2 than in fraction P_1 (Table II). As summarized in Fig. 1, the distribution of HCO_3^--ATPase and marker enzymes from the two fractions after sucrose density gradient centrifugation also supported the view that HCO_3^--ATPase in the microsomal fraction originated in plasma membrane not mitochondria. In the microsomal fraction (Fig. 1B), the HCO_3^--ATPase activity distribution virtually coincided with the 5'-nucleotidase pattern, and both were distributed very differently from cytochrome *c* oxidase. On the other hand, the HCO_3^--ATPase activity of the mitochondrial fraction behaved very differently. As seen in Fig. 1A, the distribution of HCO_3^--ATPase activity from the fraction P_1 paralleled that of cytochrome *c* oxidase, but not that of 5'-nucleotidase.

The sensitivity of HCO_3^--ATPase to oligomycin was also determined. As shown in Table III, while the HCO_3^--ATPase activity in both fractions was inhibited by oligomycin, the degree of inhibition

TABLE I

RECOVERY OF PROTEIN AND ENZYME ACTIVITIES DURING THE MICROSOMAL PREPARATIONS

The reaction mixture and the assay of enzyme were as described in Materials and Methods. Homogenate (H) was obtained from gill pairs six and seven. N, pellets from 800×*g*; P_1, pellet from 10000×*g*; P_2, pellet from 100000×*g*; S, supernatant from 100000×*g*; and %, percentage of total protein. Values are means±S.E. from nine different experiments. Specific activity is expressed as μmol/mg protein per 15 min.

Fraction	Protein		Mg^{2+}-ATPase		HCO_3^--ATPase	
	mg	%	Specific activity	%	Specific activity	%
Low salinity						
Homogenate (H)	93.8±14.7	100	2.35±0.29	100	4.40±0.57	100
Nuclear (N)	11.0± 1.7	11	3.07±0.18	15	5.19±0.79	14
Mitochondrial (P_1)	18.4± 1.8	20	6.18±0.31	52	9.58±0.45	43
Microsomal (P_2)	13.4± 2.4	14	4.36±0.61	27	11.76±1.01	38
Supernatant (S)	45.4± 4.7	48	0.03±0.01	1	0.03±0.01	<1
Total recovery		93		95		95
High salinity						
Homogenate (H)	110.0±16.9	100	1.36±0.12	100	2.24±0.23	100
Nuclear (N)	16.4± 1.8	15	1.91±0.09	21	2.95±0.72	20
Mitochondrial (P_1)	15.7± 1.7	15	4.87±0.11	51	8.57±0.58	55
Microsomal (P_2)	13.2± 1.9	12	2.62±0.14	23	5.13±0.62	28
Supernatant (S)	53.9± 8.2	49	0.02±0.01	1	0.02±0.01	1
Total recovery		91		96		102

TABLE II

INTRACELLULAR DISTRIBUTION OF MARKER ENZYMES

The reaction mixture and the assay of enzymes were as described in Materials and Methods. Values are means ± S.E. from three to six different experiments. Specific activity is expressed as μmol/h per mg protein for 5'-nucleotidase and Δ log [ferrocytochrome *c*]/min per mg protein for cytochrome *c* oxidase. %, percentage of total activities.

Fraction	Cytochrome *c* oxidase			5'-Nucleotidase		
	Specific activity	Total activity	%	Specific activity	Total activity	%
Low salinity						
H	1.69 ± 0.12	158.52	100	0.43 ± 0.09	38.46	100
N	3.91 ± 0.16	43.01	27	0.33 ± 0.07	3.63	9
P_1	7.08 ± 0.29	130.27	82	0.28 ± 0.05	5.15	13
P_2	0.31 ± 0.05	4.15	3	0.55 ± 0.07	7.37	19
S	0.02 ± 0.02	0.91	1	0.51 ± 0.14	23.15	60
Total recovery		178.34	113		39.30	101
High salinity						
H	1.39 ± 0.07	152.90	100	0.47 ± 0.12	49.50	100
N	3.39 ± 0.07	55.60	36	0.32 ± 0.09	5.25	11
P_1	5.61 ± 0.33	88.08	58	0.36 ± 0.08	5.65	11
P_2	0.34 ± 0.05	4.49	3	0.48 ± 0.07	6.34	13
S	0.08 ± 0.03	4.31	3	0.55 ± 0.15	29.65	60
Total recovery		152.48	100		46.89	95

was quite different. Stronger oligomycin inhibition of both Mg^{2+}- and HCO_3^--ATPase activities (70–90%) was observed in mitochondrial fraction (P_1) than in the microsomal fraction (50–70%) (Table III). Considered together, these results (Tables I–III and Fig. 1) indicate that contamination of the microsomal fraction by mitochondrial ATPase does not account for the HCO_3^--ATPase activity found

TABLE III

EFFECT OF OLIGOMYCIN ON ATPase ACTIVITY

The oligomycin was preincubated with enzymes in the reaction mixture at the indicated concentrations for 10 min at 30°C. Conditions for the enzyme assay were as described in Materials and Methods. Values are means ± S.E. from four to six different experiments. Specific activity is expressed as μmol/mg protein per 15 min.

Oligomycin (μg/ml)	Mitochondrial fraction				Microsomal fraction			
	Mg^{2+}-ATPase		HCO_3^--ATPase		Mg^{2+}-ATPase		HCO_3^--ATPase	
	Specific activity	%	Specific activity	%	Specific activity	%	Specific activity	%
Low salinity								
0	6.25 ± 0.38	0	9.89 ± 0.74	0	4.49 ± 0.90	0	12.66 ± 1.06	0
1.0	1.06 ± 0.35	83	1.10 ± 0.25	89	2.20 ± 0.58	51	4.05 ± 0.60	68
2.5	0.94 ± 0.36	85	0.92 ± 0.22	91	1.87 ± 0.44	58	3.85 ± 0.80	70
5.0	0.81 ± 0.22	87	0.95 ± 0.14	90	1.77 ± 0.53	61	4.05 ± 1.10	68
High salinity								
0	4.91 ± 0.21	0	8.45 ± 0.49	0	2.81 ± 0.16	0	5.53 ± 0.60	0
1.0	1.58 ± 0.32	68	2.10 ± 0.24	75	1.29 ± 0.22	54	2.31 ± 0.26	58
2.5	0.85 ± 0.18	83	1.63 ± 0.27	81	1.19 ± 0.19	58	1.97 ± 0.21	64
5.0	0.68 ± 0.20	86	1.56 ± 0.11	82	1.22 ± 0.23	56	2.31 ± 0.36	58

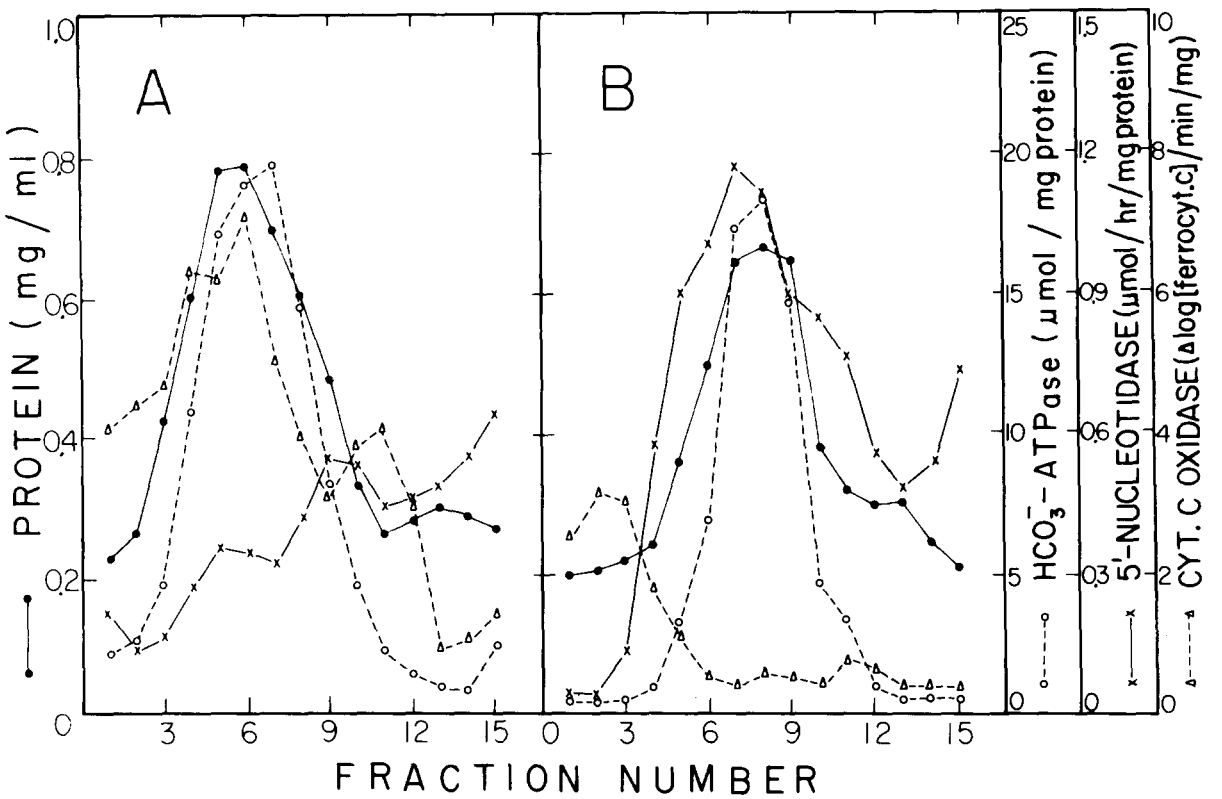

Fig. 1. Distribution of HCO_3^--ATPase and marker enzymes on a sucrose density gradient. The mitochondrial fraction, P_1(A), and the microsomal fraction, P_2(B), from low-salinity crab gills were layered over a 20–60% sucrose gradient containing 20 mM Tris-Hepes, pH 8.3, and 0.5 mM EDTA (0.8 ml of fraction containing 2.5–3.0 ml of protein). The tubes were centrifuged in a Spinco SW 50.1 rotor for 16 h at 100000× *g*. Fractions (0.4 ml) were collected by puncturing the bottom of the tube. The assay of enzymes were as described in Materials and Methods. Values are means from four different experiments.

in fraction P_2, and that at least a portion, if not all of the HCO_3^--ATPase, is of plasma membrane origin. Therefore, all experiments reported below were performed using the microsomal fraction (P_2).

In both control and low-salinity-adapted animals, the microsomal fraction contained 10–15% of the protein in the initial homogenate. Neither the pattern of recovery nor the total recovery changes significantly after low-salinity adaptation. HCO_3^--ATPase activity was markedly increased by low salinity adaptation (Table I).

HCO_3^--ATPase activity during adaptation to low salinity

When the blue crabs were transferred to low salinity (200 mosmolal) from seawater (1000 mosmolal), the HCO_3^--ATPase activity increased in all gill pairs (Fig. 2). The enzyme activity was significantly increased ($P < 0.05$) after 1 week adaptation to the low-salinity environment, and reached steady-state levels in 2 weeks (Fig. 2, inset). Gill pairs six and seven were highest in HCO_3^--ATPase activity in all animals. These gill pairs also showed the greatest increase in specific activity (approx. 2.5-fold). In subsequent experiments, gill pairs six and seven were used for characterization of the enzyme.

Properties of HCO_3^--ATPase

As noted above (Materials and Methods), the rate of ATP hydrolysis was essentially linear over the first 15 min after addition of ATP. This characteristic was unchanged in low-salinity animals. The pH optimum of the enzyme was examined in

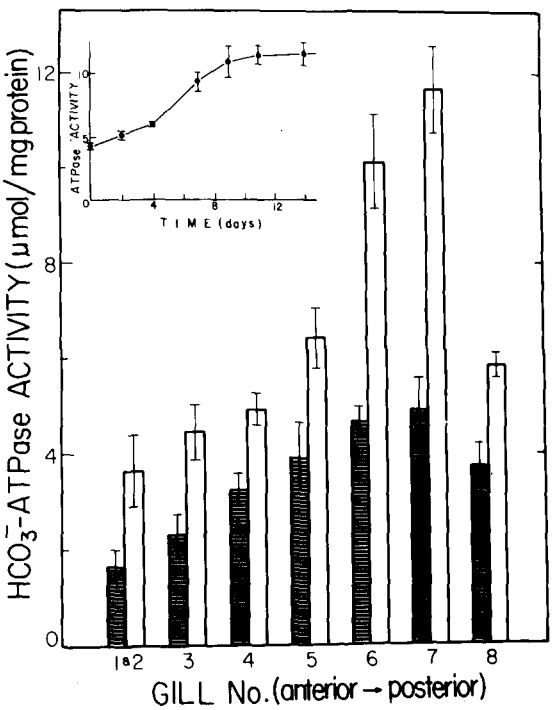

Fig. 2. Changes in HCO_3^--ATPase activity during adaptation of seawater blue crabs to low salinity. The reaction mixture and the assay of enzymes were as described in Materials and Methods. Values are means±S.E. from four to six different experiments. Striped bar, seawater; open bar, low salinity. The inset shows the time course of enzyme activity during the adaptation to low salinity. In all gill pairs HCO_3^--ATPase activity was significantly higher ($P < 0.05$) in animals acclimated to low salinity.

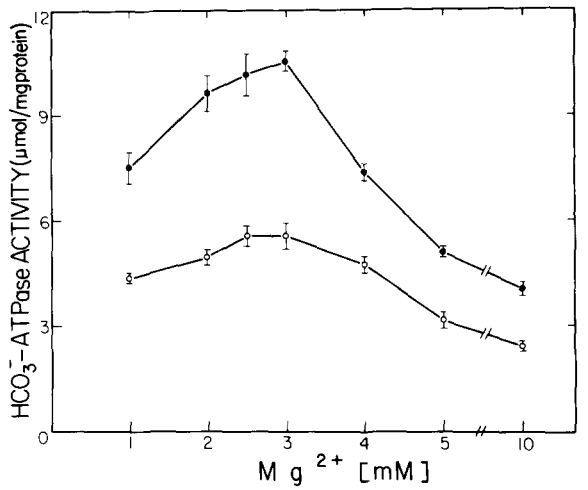

Fig. 3. Effect of Mg^{2+} on the HCO_3^--ATPase activity. The reaction mixture and the assay of enzyme were as described in Materials and Methods, except the concentrations of Mg^{2+} were varied as indicated above. Values are means±S.E. from three different experiments. ●——●, low salinity; ○——○, seawater.

both groups of animals over a range from pH 6.5 to 8.3. Maximum enzyme activity was observed at pH 7.8 in both groups. Finally, as shown in Fig. 3, HCO_3^--ATPase activity was optimal at 3 mM Mg^{2+} ($MgCl_2$ or $MgSO_4$) in both groups.

The substrate specificity of the enzyme was examined using several nucleotides (Table IV). ATP was the most effective nucleotide. GTP and ITP were also effective, but CTP, UTP and XTP were not effectively hydrolyzed. ADP, AMP and the ATP analogoues, 5'-adenylyl imidodiphosphate (Ado*PP*[NH]*P*), were not hydrolyzed by the ATPase. As seen for the other properties described above, no change was observed in the specificity of enzyme from the low-salinity animals even though their HCO_3^--ATPase activity was 2.3-times that of the high-salinity controls.

Kinetic properties of HCO_3^--ATPase

HCO_3^-. Bicarbonate was added to the reaction mixture as $NaHCO_3$. An Na^+ effect could be ruled out because NaCl at concentrations up to 100 mM had no appreciable effect on the ATPase activity in the absence of $NaHCO_3$. As shown in Fig. 4, maximal HCO_3^--ATPase activity was achieved at 50 mM $NaHCO_3$ in crabs from low-salinity and full-strength seawater. HCO_3^- concentrations higher than 50 mM resulted in reduced enzyme activity. The apparent K_a for HCO_3^- in microsomes from both groups, i.e., high- and low-salinity animals, was determined from Lineweaver-Burk plots (Fig. 4, inset). The plots were linear, and the apparent K_a was found to be the same (8.9 mM) in both. Thus, the affinity of the enzyme system for HCO_3^- was not altered during the adaptation.

ATP. Lineweaver-Burk plots for the microsomal HCO_3^--ATPase with ATP as substrate were linear, and the observed K_m for ATP was 4.4. mM in both salinities (Fig. 5). This result indicated that low-salinity adaptation increased the V_{max} from 9.6 to 25.6 µmol/mg protein per 15 min without altering the apparent K_m for ATP.

Temperature. In order to define a possible

TABLE IV

SUBSTRATE SPECIFICITY OF HCO_3^--ATPase

The enzyme activity was assayed for its ability to liberate P_i from various nucleotides (5 mM) in the presence of Mg^{2+}. Conditions for the assay were as described in Materials and Methods. Values were means ± S.E. from four different experiments. Specific activity is expressed as μmol/mg protein per 15 min.

Substrate	Low salinity		High salinity	
	HCO_3^--ATPase specific activity	% of rate with ATP	HCO_3^--ATPase specific activity	% of rate with ATP
ATP	11.0 ± 1.1	100	4.7 ± 0.2	100
ITP	9.4 ± 1.0	86	4.3 ± 0.3	92
GTP	9.3 ± 1.0	85	3.8 ± 0.2	81
XTP	2.9 ± 0.8	26	1.1 ± 0.1	23
UTP	2.4 ± 0.5	22	1.0 ± 0.3	21
CTP	2.0 ± 0.9	18	0.7 ± 0.3	15
Ado*P*[NH]*P*	0.9 ± 0.3	8	0.1 ± <0.1	2
ADP	0.1 ± <0.1	1	0	0
AMP	0	0	0	0

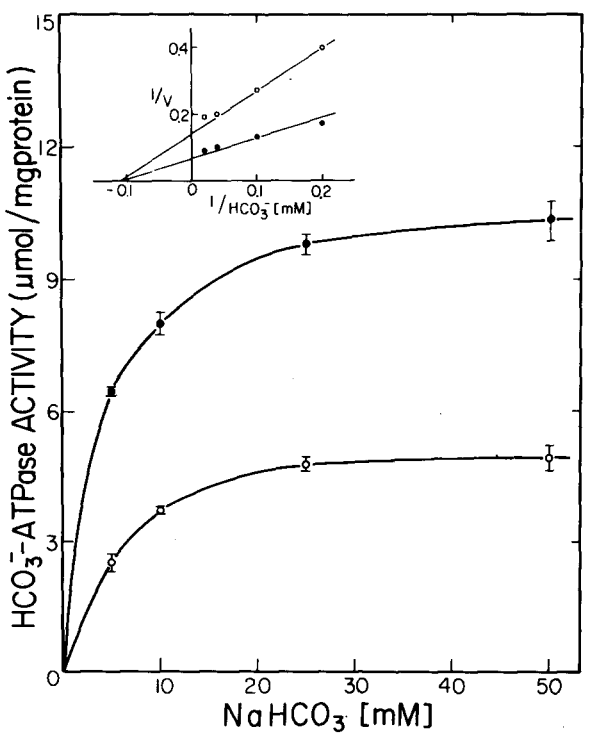

Fig. 4. Effect of HCO_3^- on ATPase activity. The experimental conditions were as described in Materials and Methods, except the concentrations of $NaHCO_3$ were as indicated above. Values are means ± S.E. from four different experiments. ●——●, low salinity; ○——○, seawater. The inset shows Lineweaver-Burk plots for HCO_3^--ATPase activity.

mechanism for the increased HCO_3^--ATPase activity in low-salinity animals, the effect of temperature on the enzyme activity was studied. As shown in Table V, the V_{max} of HCO_3^--ATPase in both low- and high-salinity animals increased with in-

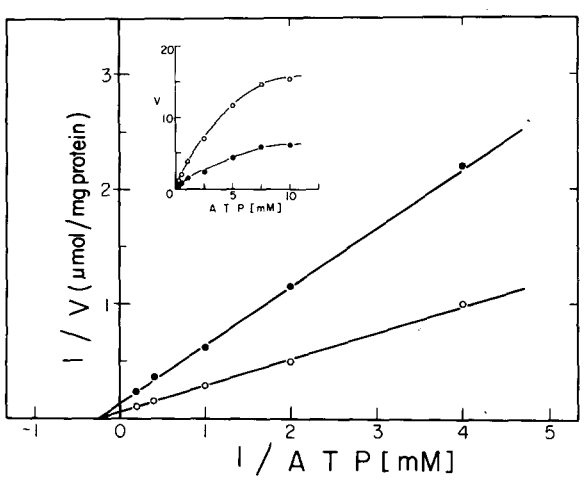

Fig. 5. Lineweaver-Burk plots for HCO_3^--ATPase activity. The experimental conditions were as described in Materials and Methods, except the concentrations of ATP were varied as indicated above. Values are means ± S.E. from three different experiments. ●——●, seawater; ○——○, low salinity. The inset shows the enzyme activity with different concentrations of ATP.

TABLE V

EFFECT OF TEMPERATURE ON THE KINETIC PROPERTIES OF HCO_3^--ATPase WITH ATP AS THE VARIABLE SUBSTRATE

The reaction mixture and the assay of enzyme were as described in Materials and Methods. Values are means from three different experiments, and range of the values are given in parentheses. All crabs were acclimated to 16°C. Specific activity is expressed as μmol/mg protein per 15 min.

Temperature (°C)	Low salinity		High salinity	
	K_m (mM)	V_{max} (specific activity)	K_m (mM)	V_{max} (specific activity)
13	3.9 (4.6–3.6)	8.4 (9.4–7.3)	4.5 (4.6–3.5)	4.2 (4.6–3.8)
30	4.4 (5.3–4.1)	25.6 (31.2–21.0)	4.2 (4.4–3.7)	9.6 (11.1–7.8)
37	4.2 (5.9–3.8)	33.3 (34.5–25.1)	4.8 (5.6–4.3)	10.0 (15.0–8.9)

creasing temperature, whereas the K_m for ATP did not change. The enzyme activity from seawater crabs, however, never reached the levels shown in low-salinity animals at any of the temperatures tested, suggesting that the increased enzyme activity was not due to a modulation of the membrane-bound enzyme by membrane fluidity.

Effect of inhibitors

To investigate possible factors involved in the HCO_3^--ATPase activity, the effects of various inhibitors were studied. As shown in Fig. 6, NaSCN effectively inhibited the HCO_3^--ATPase activity in microsomal preparations from low- and high-salinity animals. The I_{50} for NaSCN was 4.8 mM for both groups. The effects of several other inhibitors on enzyme activity are shown in Table VI. Acetazolamide, a carbonic anhydrase inhibitor, inhibited 60% (4 mM) to 85% (5 mM) of the HCO_3^--ATPase activity. Inhibitors of other membrane-bound ATPase activities were not effective HCO_3^--ATPase inhibitors. Ouabain ($(Na^+ +$

TABLE VI

EFFECT OF INHIBITORS ON HCO_3^--ATPase ACTIVITY

The inhibitors were preincubated with enzymes in the reaction mixture at the indicated concentrations for 10 min at 30°C. Conditions for the enzyme assay were as described in Materials and Methods. Values are means ± S.E. from four different experiments. Specific activity is expressed as μmol/mg protein per 15 min.

Inhibitor	Concentration (mM)	HCO_3^--ATPase (specific activity)	Inhibition (%)
None		10.69 ± 0.98	0
Acetazolamide	4	4.27 ± 0.09	60
	5	1.56 ± 0.03	85
Ouabain	1	11.01 ± 1.00	0
	5	9.88 ± 0.96	8
Vanadate	0.1	10.80 ± 0.92	0
	0.5	9.30 ± 1.02	13
EDTA	4	2.43 ± 0.02	77
	5	0.62 ± 0.01	94
PCMBS	0.1	1.83 ± 0.01	83
	0.5	1.32 ± 0.01	88
+ dithiothreitol (0.5 mM)	0.1	10.53 ± 0.28	2
+ dithiothreitol (1.0 mM)	0.5	11.21 ± 0.62	0

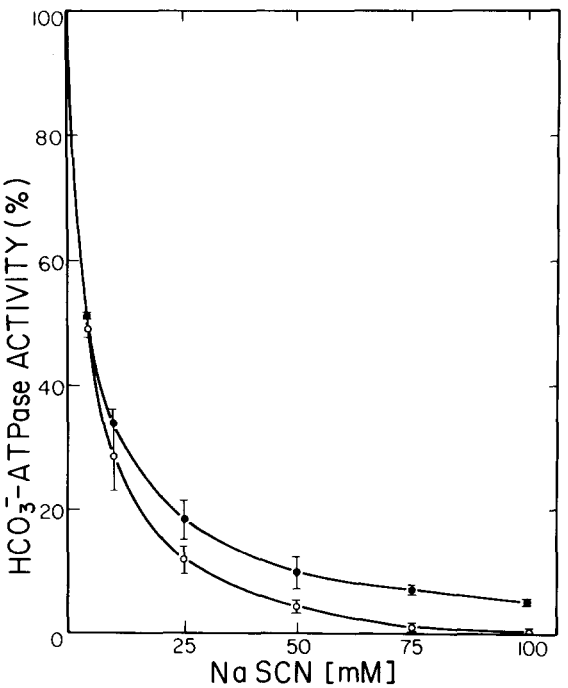

Fig. 6. Effect of NaSCN on HCO_3^--ATPase activity. NaSCN was preincubated with enzyme in the reaction mixture at the indicated concentrations for 10 min at 30°C. Conditions for the enzyme assay were as described in Materials and Methods. Values are means±S.E. from four different experiments. ●——●, low salinity; ○——○, seawater.

K^+)-ATPase inhibitor) and vanadate (inhibits both ($Na^+ + K^+$)- (and Ca^{2+}-ATPases) produced less than 15% inhibition even at relatively high doses. EDTA completely abolished enzyme activity at 5 mM, presumably by chelating Mg^{2+} although a direct effect on the enzyme cannot be ruled out. The sulfhydryl inhibitor, PCMBS, greatly reduced the HCO_3^--ATPase activity. This effect was completely reversed by dithiothreitol.

Effect of Triton X-100 on solubilization of HCO_3^--ATPase

The addition of Triton X-100 (1% final concentration) effectively solubilized the microsomal HCO_3^--ATPase (Table VII). When the microsomes from seawater crab gills were treated with Triton X-100, the total solubilized enzyme activity, and its specific activity, did not reach the activity level seen after adaptation to low salinity. This result again suggested that the alteration of enzyme activity was not due to a modulation of membranes or of the existing enzyme activity.

Discussion

The results presented above demonstrate that an HCO_3^--stimulated and SCN^--inhibited Mg^{2+}-ATPase is present in microsomal preparation of blue crab gills (Tables I and II). Furthermore, in contrast to the observations of Van Amelsvoort et al. [12] in rainbow trout grill and Ho and Chan

TABLE VII

EFFECT OF TRITON X-100 ON SOLUBILIZATION OF HCO_3^--ATPase

Microsomal preparations (P_2) were mixed with Triton X-100 at final concentrations of 1% and incubated at 4°C for 60 min. The mixture was centrifuged at 100000×*g* for 60 min and the supernatant and pellets were assayed as described in Materials and Methods. Values are means±S.E. from three different experiments. Specific activity is expressed as μmol/mg protein per 15 min.

Fraction	Protein (mg)	HCO_3-ATPase (specific activity)	Total activity (μmol/15 min)
Low salinity			
P_2	10.0	10.5±1.1	105.0
Triton X-100 supernatant	6.1±0.5	19.3±2.6	117.7
Triton X-100 pellets	5.4±0.4	0.2±0.1	0.8
High salinity			
P_2	10.0	5.4±0.5	54.0
Triton X-100 supernatant	5.9±0.5	10.7±1.4	63.1
Triton X-100 pellets	5.2±0.3	0.1±0.0	0.5

[15] in Japanese eel gill, they indicate (Tables I and II and Fig. 1) that the origin of this HCO_3^--ATPase activity is not totally mitochondrial. These observations are similar to those of Kerstetter and Kirschner [10], Bornancin et al. [16] and De Pew and Towle [19] who concluded that an anion-dependent ATPase exists in microsomal fractions of rainbow trout gills and in plasma membrane fractions of fiddler crab gills. This HCO_3^--ATPase increased markedly when the animals were transferred from full-strength seawater to low salinity. The maximal enzyme activity was observed at an Mg^{2+} concentration of 3 mM, and pH 7.8. Cl^- did not activate the ATPase. The optimal HCO_3^- concentration was 50 mM, identical to the optimum concentration for HCO_3^--ATPase from rainbow trout gill [10] and rat kidney cortex [38]. The apparent K_a for HCO_3^- was 8.9 mM, a value somewhat lower than the K_a of HCO_3^--ATPase in the gills of rainbow trout (16 mM) [10]. The substrate specificity of blue crab gill HCO_3^--ATPase was also found to be similar to that for teleost and mammalian enzyme [5,11]. ATP was the most effective nucleotide of those tested, and the apparent K_m for ATP was 4.4 mM (Fig. 5). HCO_3^--ATPase activity was effectively inhibited by acetazolamide, EDTA and PCMBS whereas ouabain or vanadate did not inhibit. Thus, the overall properties of the blue crab gill HCO_3^--ATPase appear to be similar to those of the teleosts and mammals.

The crab gill HCO_3^--ATPase was effectively inhibited by NaSCN; the I_{50} was 4.8 mM in microsomal preparations from both low- and high-salinity-adapted animals (Fig. 6). The NaSCN sensitivity of the crab HCO_3^--ATPase was somewhat lower than that of Triton X-100-extracted enzymes from the gills of rainbow trout [10] or the intestinal mucosa of the eel [11] where maximal inhibition was seen at a concentration of 5 mM. In experiments designed to assess the role of HCO_3^--ATPase in ion transport, Kristensen [39] showed that SCN^- inhibited active Cl^- transport across frog skin without interfering with Na^+ transport. In addition, Epstein et al. [40] showed that a low concentration of SCN^- (0.15 mM) in the external medium inhibited Cl^- influx [60%] into freshwater goldfish, while Na^+ uptake was unchanged. Kerstetter and Kirschner [10] suggested a possible relationship between the HCO_3^--ATPase and Cl^- transport in rainbow trout gills. They showed that both HCO_3^--ATPase activity and Cl^- uptake by intact gills were inhibited by SCN^-, but apparently by independent mechanisms, because Cl^- was not required for activation of the ATPase. In fact, Cl^- inhibited the enzyme at concentrations above 10 mM.

The carbonic anhydrase inhibitor, acetazolamide, inhibited the blue crab HCO_3^--ATPase activity (Table VI). Maetz [41] showed that injection of acetazolamide inhibited Na^+ and Cl^- uptake by intact crab gills and suggested that the catalytic hydration of respiratory CO_2 provided H^+ and HCO_3^- for Na^+-H^+ and Cl^--HCO_3^- exchange across the gill epithelium [42,43]. Kerstetter et al. [44] confirmed that injection of acetazolamide inhibits Na^+-H^+ exchange but not Cl^- uptake in the trout and suggested that HCO_3^- diffusing from the blood into the epithelial cells was readily available and, therefore, not rate limiting for Cl^--HCO_3^- exchange at the apical border. These investigators attributed the effect of acetazolamide solely to its inhibition of carbonic anhydrase; however, the present data indicate a possible direct effect on HCO_3^--ATPase activity as well. Thus, the data available at present are consistent with the hypothesis that the increased HCO_3^--ATPase activity in the gills may be involved with active Cl^- uptake.

One additional approach to this question was examined in this paper. If HCO_3^--ATPase were involved with active ion transport, it should presumably increase when Cl^--HCO_3^- exchange is increased. Thus, it should increase in gills when the blue crabs are adapted to low salinities, just as $(Na^+ + K^+)$-ATPase does. Indeed, this was the case (Fig. 2). The increased crab gill HCO_3^--ATPase activity usually appeared after 4 days adaptation to low salinity, and reached a steady-state level by 2 weeks. The greatest increase in the HCO_3^--ATPase activity occurred in the posterior region of the gill chamber, particularly in the sixth and seventh gill pairs. A similar increase has been observed in the gill $(Na^+ + K^+)$-ATPase of crabs [31]. Kinetic experiments showed that low-salinity adaptation increased the V_{max} without altering the apparent K_m for ATP (Fig. 5). Similarly, the apparent K_a for HCO_3^- was also found to be the same in both salinities (Fig. 3). Thus, at least the

nature of the response is correlated with the osmotic stress and need for increased ion transport. However, the slow time course of adaptation raises the question of whether or not this newly stimulated ATPase has a role in rapid osmotic and ionic regulation seen after transfer to low salinity, or whether it may be related to regulation of cellular composition alone. This picture is very similar to that for gill ($Na^+ + K^+$)-ATPase, where activity was increased after exposure to low salinity and the extent of increase is proportional to the degree of osmotic stress, but the appearance of increased activity shows a much slower time course than the establishment of new hemolymph osmolarity [31]. This fact may indicate either that gill ATPases are not the only controlling factors in osmoregulation, or as Towle et al. [27] have suggested, that existing ATPase activities may be modulated in response to osmotic stress. However, for HCO_3^--ATPase it appears that the increase in activity reflects synthesis of new enzyme not activation. When the microsomal HCO_3^--ATPase from seawater blue crab gills (a) was solubilized with Triton X-100 (Table VII) or (b) assayed at different temperatures (Table V), the total enzyme activity of seawater animals, as well as the specific activity, did not reach the level seen from the low-salinity crabs. These results are consistent with de novo synthesis of HCO_3^--ATPase during low-salinity adaptation rather than modulation of membranes or of the existing enzyme activity.

From overall considerations, the HCO_3^--ATPase in the gills, like HCO_3^--ATPase from other transporting epithelia, appears likely to play an important role in anion transport. However, conclusive evidence that the HCO_3^--ATPase is involved with either osmoregulation or acid-base balance [16,17] will require further experimentation. The blue crab gill enzyme may be an excellent model system with which to approach these questions. First, the enzyme can be induced to a relatively high specific activity by low-salinity adaptation, and second, the enzyme can be readily solubilized. Thus, proteoliposomes may be prepared in which to study the details of the relationships between the ATPase and ion transport.

Acknowledgements

The author would like to thank Dr. J.B. Pritchard for his generous continuing support and help with the manuscript, Dr. J.L. Renfro for helpful discussion, and F.R. Kulpa for excellent technical assistance. I thank Mrs. Rena Greene for assistance with the typing and preparation of the manuscript.

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