Characterization of transport Na\(^+\)-ATPases in gills of freshwater tilapia
Evidence for branchial Na\(^+\)/H\(^+\) (−NH\(_4^+\)) ATPase activity in fish gills

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Abstract
Branchial plasma membranes from the freshwater cichlid teleost Oreochromis mossambicus (tilapia) contain two Na\(^+\)-dependent ATPases: Na\(^+\)/K\(^+\) ATPase, and an amiloride-sensitive ATPase which is postulated to operate as a Na\(^+\)/H\(^+\) (−NH\(_4^+\)) ATPase. It is suggested that both enzyme activities are located in the basolateral membrane system of the chloride cells. K\(^+\) has opposing effects on the two enzymes: it stimulates Na\(^+\)/K\(^+\) ATPase and inhibits Na\(^+\)/H\(^+\) (−NH\(_4^+\)) ATPase activity. Na\(^+\)/H\(^+\) ATPase appears more sensitive to NH\(_4^+\) ions at low concentrations than Na\(^+\)/K\(^+\) ATPase and the stimulatory effect by NH\(_4^+\) ions on the first enzyme could be important in facilitating NH\(_4^+\) excretion by tilapia gills under physiological conditions. In vitro maximum stimulation by NH\(_4^+\) is similar for the two enzymes (200%). In contrast to Na\(^+\)/K\(^+\) ATPase, Na\(^+\)/H\(^+\) ATPase activity is inhibited by supra-physiological (> 20 mM) concentrations of NH\(_4^+\).

Introduction
Freshwater teleosts continuously take up sodium ions from the strongly hypotonic environment in order to compensate for the diffusional loss of the cation. The gills have been shown to be the major organ in the uptake process. The chloride cells (Keys and Wilmer 1932), or ionocytes, are generally considered as the actual sites of Na\(^+\) uptake in the tight epithelium of the gills. This assumption was at first based on the observation that these cells contain high concentrations of mitochondria. More recently it was established, both by ultracytochemical (Hootman and Philpott 1979) and biochemical (Sargent et al. 1975a) evidence, that the chloride cells contain the highest levels of Na\(^+\)/K\(^+\) ATPase of all branchial cells. In contrast to suggestions put forward by several authors (reviewed by Payan et al. 1984) that under freshwater conditions lamellar respiratory cells are the main sites of Na\(^+\) influx, recent evidence seems to reinstate the chloride cells as the sites of active Na\(^+\) uptake in freshwater (Avella et al. 1987). To date, the electrochemical gradient of Na\(^+\) which is built up via Na\(^+\)/K\(^+\) ATPase activity is regarded as the driving force for transepithelial Na\(^+\) uptake in a variety of ion translocating epithelia (Skou 1973; Kirschner 1983; Jorgensen 1985), including fish gills (de Renzis and Bornancin 1984). Studies on the localization and biochemical characteristics of branchial Na\(^+\)/K\(^+\)

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ATPase for a number of species have shown that the teleost enzyme meets the requirements for a Na\(^+\) transport enzyme in freshwater: its basolateral localization in the chloride cells (Hootman and Philpott 1979) agrees with the idea that the energy-requiring step in transepithelial Na\(^+\) transport under freshwater conditions is the extrusion of sodium ions from the chloride cells into the extracellular space.

In order to maintain electrical neutrality, fish must balance their net Na\(^+\) uptake by excreting equal amounts of positive charge. Flux studies with intact fish have shown that Na\(^+\)/H\(^+\) exchange is the most likely mechanism operating at the gill level in freshwater. Exchange of Na\(^+\) for H\(^+\) has been shown to be important in Na\(^+\) uptake (Kirschner 1973; Maetz 1973) but the concomitant excretion of protons is an important factor in acid-base regulation (Heisler 1984a). The Na\(^+\)/H\(^+\) exchange mechanism seems to have evolved early in vertebrate history, that is, before representatives of the group entered freshwater (Evans 1984a). To date information on the molecular basis for Na\(^+\)/H\(^+\) exchange is scarce. Na\(^+\)/H\(^+\) antiporters have been described in eukaryotes as well as in prokaryotes (Krulwich 1983). In higher vertebrates Na\(^+\)/H\(^+\) exchange occurs in several epithelia (Forte and Reenstra 1985) and, dependent on the tissue studied, electrogenic proton pumps or electroneutral Na\(^+\)/H\(^+\) exchange mechanisms operate. From the data of Ehrenfeld et al. (1985) it appears that an electrogenic active proton pump facilitates Na\(^+\)/H\(^+\) exchange at the apical membrane of the skin of the amphibian *Rana esculenta*. Several authors studying the coupling of Na\(^+\) uptake and H\(^+\) extrusion in gills of freshwater teleosts, however, have envisaged an electroneutral antiport system located in the apical membrane of the chloride cells driven by the basolateral Na\(^+\)/K\(^+\) ATPase (Kerstetter et al. 1970; Maetz 1974). Evidence for the existence of an apically located Na\(^+\)/H\(^+\) antiporter in part stems from studies applying the diuretic amiloride (Kirschner 1973), which blocks Na\(^+\) channels at low concentrations (< 10 \(\mu\)M) but, at higher concentrations, also inhibits Na\(^+\)/H\(^+\) exchange mechanisms (Benos 1982). Different approaches have been applied to study the effect of amiloride on Na\(^+\) uptake in freshwater fish. In an *in vivo* study Kirschner et al. (1973) observed that amiloride, when added to the external medium, inhibited Na\(^+\) influx and H\(^+\) efflux, leaving Na\(^+\) efflux — which occurs via paracellular routes — and both Cl\(^-\) influx and efflux unaffected. Using an isolated trout gill preparation and rather high concentrations (2 mM) of the diuretic, Kerstetter and Keeler (1976) confirmed the inhibitory action of amiloride on Na\(^+\) uptake. Finally, Avella et al. (1985), studying the effect of amiloride on Na\(^+\) uptake by the isolated trout head, observed a rapid reduction (70% in less than 1 min) in the Na\(^+\) influx when 0.1 mM amiloride was added to the external medium. Essentially, these studies concentrated on apically located Na\(^+\)/H\(^+\) exchange mechanisms. In view of the strong correlation observed between transepithelial Na\(^+\) uptake and H\(^+\) efflux in freshwater fish, we decided to investigate whether in teleost gills Na\(^+\) could be exchanged for H\(^+\) in both steps of the transepithelial transport, i.e., both apically and basolaterally. Since it can be anticipated that the basolateral translocation of Na\(^+\) is energy dependent, this would imply that the basolateral membrane system contains Na\(^+\)/H\(^+\) ATPase as well as Na\(^+\)/K\(^+\) ATPase activity. Interestingly, several authors have reported K\(^+\)-independent, Na\(^+\)-activated ATPase activities in the gills of teleosts (Pfeifer and Kirschner 1972; Pfeifer 1978; Borgatti et al. 1985). However, the function of these Na\(^+\)-dependent enzyme activities in fish gills could not be elucidated. In this communication we investigated the possibility of more than one Na\(^+\)-dependent ATPase activity functioning in branchial Na\(^+\) transport by studying the effects of both ouabain (Na\(^+\)/K\(^+\) ATPase inhibitor) and amiloride (Na\(^+\)/ H\(^+\) exchange inhibitor) on Na\(^+\)-activated ATPase activity in purified gill plasma membranes of the cichlid teleost *Oreochromis mossambicus* (tilapia).

**Materials and methods**

**Animals**

Female tilapia (body weight about 20 g) were ob-
Purification of plasma membranes

Animals were killed by spinal transection. Gill bars were removed and the soft tissue scraped off onto a glass plate (0°C). Material was collected in an isotonic buffer containing: 5 mM imidazole/L-histidine pH 7.5, 250 mM sorbitol, 12.5 mM KCl, 0.2 mM PMSF and 0.5 mM Na₂EDTA. The original homogenate (H₀) was prepared by 12 strokes with a loosely fitting dounce homogenizer in a large volume of buffer. Further purification was achieved by differential centrifugation: 50 min at 100,000 × g (Beckman L8-80) yielded a pellet (P₀) which was resuspended in 12 ml of medium by 100 douncer strokes. Nuclei were discarded (P₁) after 10 min at 1,000 × g (Beckman TL-6) and a pellet mainly containing mitochondria (P₂) was obtained after 10 min at 10,000 × g (Sorvall RC-513). A 60 min run at 100,000 × g (Beckman L8-80) resulted in a final pellet (P₃), which consisted of leaky (EDTA) plasma membrane vesicles and fragments. Pellets P₂ and P₃ were resuspended in small volumes of ultrapure water, diluted to 0.5 mg protein/ml, quickly frozen in liquid nitrogen and stored at -70°C. Enzyme activities usually did not decline within one week. The K⁺ concentration in the stored plasma membrane fraction was approximately 6 μM.

Assays

Membrane protein was measured using a commercial BioRad protein kit (500–0006) with bovine serum albumin (BSA) as reference. Na⁺/K⁺ ATPase activities were determined by the method of Bonting and Caravaggio (1963); 10 μg of protein was added to 380 μl of medium containing: 100 mM NaCl, 5 mM MgCl₂, 0.1 mM H₂EDTA, 3 mM Na₂ATP (vanadate free, Sigma) and 30 mM imidazole pH 7.4. Enzyme activity was calculated from the difference in ATP hydrolysis in medium A (12.5 mM KCl added) and medium E (1 mM ouabain added), after incubating 10 min at 37°C. The K⁺ concentration in medium E amounted to about 11 μM. Reactions were stopped on ice by adding 1.5 ml of 8.6% TCA, and the amount of inorganic phosphate (P₂) liberated from ATP (determined according to Fiske and Subbarow 1925) was used for ATPase activity calculations. No detergents were used. Activities are expressed as μmoles P₂/h/mg protein. Amiloride inhibition of Na⁺-dependent ATPase activity was calculated from the difference in ATPase activities between media with or without 3 mM amiloride. Basal ATPase activity (i.e. insensitive to ouabain and amiloride) consisted of aspecific phosphatase activity. Succinate dehydrogenase (SDH) activity was estimated according to Pennington (1961), using fresh material. Activities are expressed as Δ A₄₉₀/h/mg protein.

All chemicals were of highest quality commercially available; amiloride was a gift from Merck, Sharpe & Dohme.

Results

With our isolation procedure plasma membrane fractions were obtained that were highly enriched in Na⁺/K⁺ ATPase. The specific activity of the P₃ fraction was 11 fold higher than in the original homogenate (H₀; Table 1). The low recovery (0.4%) of SDH activity indicated that mitochondrial contamination was negligible. On the other hand, almost 20% of the Na⁺/K⁺ ATPase activity was recovered. Figure 1 demonstrates that the plasma membrane fraction of tilapia gills contained an amiloride sensitive Na⁺-ATPase entity distinct from Na⁺/K⁺ ATPase. Due to the high endogenous K⁺ concentrations in the original fractions (H₀), it was impossible to measure amiloride sensitive Na⁺-ATPase entity reliably in the H₀, and therefore the purification factor (ratio specific activity P₃/H₀) could not be determined for this enzyme. However, the enzyme activities in the subsequent fractions (P₀, S₁, S₂ and P₃) strongly suggested that the two Na⁺-ATPase activities were co-purified. The two Na⁺-ATPase fractions appeared to have some properties in common (Table 2): pH optimum, the high preference for ATP as a
Table 1. Comparison of succinic dehydrogenase (SDH) activity and Na\(^+\)/K\(^+\) ATPase activity in the original homogenate (H\(_0\)), the mitochondrial pellet (P\(_2\)) and the plasma membrane fraction (P\(_3\)) of tilapia gills

<table>
<thead>
<tr>
<th></th>
<th>Protein recovery %</th>
<th></th>
<th></th>
<th>SDH</th>
<th>Na(^+)/K(^+) ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_0)</td>
<td>100.0</td>
<td>1544</td>
<td>1</td>
<td>6485</td>
<td>100.0</td>
</tr>
<tr>
<td>P(_2)</td>
<td>7.7</td>
<td>2780</td>
<td>1.8</td>
<td>890</td>
<td>13.7</td>
</tr>
<tr>
<td>P(_3)</td>
<td>1.6</td>
<td>375</td>
<td>0.2</td>
<td>26</td>
<td>0.4</td>
</tr>
</tbody>
</table>

SDH activities in ΔA\(_{490}\)/h/mg protein (specific activity); Na\(^+\)/K\(^+\) ATPase activities in μmol Pi/h/mg protein (specific activity).

Fig. 1. Amiloride inhibition of Na\(^+\) dependent, K\(^+\) independent ATPase activity in gill plasma membranes of tilapia. The specific ATPase activity (μmol Pi/h/mg protein) in the A — K\(^+\) medium was taken as 100%. Different concentrations of amiloride were added to this medium. 'A + K': assay medium 'A' (for composition see Materials and methods) containing 12.5 mM K\(^+\) (as KCl). 'A — K': assay medium 'A' without K\(^+\). 'E': assay medium 'A' with 1 mM ouabain added. Results are shown as the means (± SEM) of three experiments.

Table 2. Comparison of ouabain sensitive and amiloride sensitive sodium dependent ATPase activities in gill plasma membranes of tilapia

| pH optimum | 7.85 | 8.00 |
| Substrate specificity: | ADP/ATP activ. ratio | 0.20 | 0.31 |
| AMP/ATP activ. ratio | 0.03 | 0.05 |
| Activation energy* | 22 Kcal/mole | 16 Kcal/mole |
| Oligomycin sensitivity** | absent | absent |
| K\(_a\) Mg\(^++\) | 0.65 mM | 0.45 mM |
| K\(^+\) effect (concentration for ½ max. effect) | (7.4 mM) | (5.7 mM) |

* In both cases, no discontinuity was observed in the range from 10°C—37°C; ** Oligomycin was tested in concentrations ranging from 1 μg/ml to 15 μg/ml.

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Discussion

The results presented in this paper demonstrate for
Fig. 2. $\text{NH}_4^+$ stimulation of ouabain-sensitive (Na$^+$/K$^+$ ATPase) and amiloride-sensitive (Na$^+$/H$^+$ ATPase) Na$^+$-ATPase activities in gill plasma membranes of tilapia. The specific activity in the absence of NH$_4^+$ was taken 100%. Values are means of three separate experiments. Half maximal stimulation for ouabain-sensitive Na$^+$-ATPase at 7 mM, and for amiloride-sensitive Na$^+$-ATPase activity: 1 mM NH$_4^+$.

the first time that in gill plasma membranes of freshwater fish amiloride sensitive Na$^+$-ATPase activity is present. This ATPase activity differed in several aspects from Na$^+$/K$^+$ ATPase.

Na$^+$/K$^+$ ATPase

The value for freshwater tilapia gill specific Na$^+$/K$^+$ ATPase activity presented in this paper is the highest reported to date: specific activities in fish literature range from 1.5 $\mu$moles P$_i$/h/mg protein (Dharmamba et al. 1975) to 14.4 $\mu$moles P$_i$/h/mg protein (Ho and Chan 1980). These differences most likely result from the isolation procedures used: both the lowest (1.5) and the highest (65.9, this paper) specific Na$^+$/K$^+$ ATPase activities published for freshwater teleost gill are for the same species, *Oreochromis mossambicus* (tilapia). Although species differences probably exist, factors inherent to the plasma membrane isolation and ATPase assay procedures used, i.e. the addition of detergents (Busacker and Chavin 1981; compare Jacob and Taylor 1983 with Towle et al. 1977), preincubation of the membrane suspensions (Trigari et al. 1985; Pfeiler and Kirschner 1972; Pfeiler 1976, 1978), *in vitro* assay temperature (Sargent et al. 1975b), the difference of this temperature with the ambient water temperatures of the fishes, and sensitivity of the membrane preparations to storage (Dharmamba et al. 1975; Johnson et al. 1977) certainly contribute the larger part to the differences observed.

The inorganic phosphate concentration in the assay medium can be an inhibiting factor of enzyme activity; however, under the assay conditions employed this concentration never exceeded 0.5 mM, which at pH 7.4 has been found without effect on Na$^+$/K$^+$ ATPase activity (Huang and Askari 1984). K$^+$ is often omitted from the ouabain medium in Na$^+$/K$^+$ ATPase assays (Schuurmans Stekhoven and Bonting 1981). This approach is valid only if the membrane preparation does not contain potassium dependent, sodium independent ATPase activity. This type of ATPase activity was found to be absent in our preparation (data not shown) and we therefore omitted K$^+$ from the ouabain medium.

The properties of tilapia gill Na$^+$/K$^+$ ATPase reported here are in agreement with previous results on teleost gill Na$^+$/K$^+$ ATPase: a pH optimum of 7.85 is close to the value of 7.6 reported for goldfish (Busacker and Chavin 1981), while the activation energy is relatively high when compared to literature data (range: 7.3 Kcal/mol for sea bass to 19.5 Kcal/mol for seawater trout). It has been mentioned that this parameter of the enzyme is extremely sensitive to storage (Pfeiler 1978). No discontinuity in the Arrhenius plot for branchial Na$^+$/K$^+$ ATPase could be observed. This is in contrast with the situation for eel (Sargent et al. 1975b, Thomson et al. 1977), but confirms results of Pfeiler on seawater trout and Trigari et al. (1985) on sea bass (range: 5°C–45°C). Shifts in enzyme activity normally are associated with changes in phospholipid viscosity of the membrane. The value for half maximal *in vitro* stimulation of Na$^+$/K$^+$
ATPase by K+ (7.4 mM) corroborates results obtained by several authors; $K_a$ values range from 0.8 mM (Pfeiler and Kirshner 1972) to 14 mM (Johnson et al. 1977). The same holds for the Mg++ concentration stimulating tilapia Na+/K+ ATPase half maximally: the value of 0.65 mM found for tilapia is close to the 0.8 mM for goldfish (calculated by us from Busacker and Chavin 1981).

**Na+/H+ ATPase**

Na+/K+ ATPase is generally accepted as the enzyme mediating active sodium uptake by a number of epithelia (Skou 1973; Kirshner 1983; Jorgensen 1985). Our present results indicate that a second Na+-dependent ATPase might be present in teleost gills, and on the basis of its amiloride sensitivity and K+ independency we postulate that this enzyme might be mediating energy dependent Na+/H+ exchange in the teleost gill. Since the enzyme co-purified with Na+/K+ ATPase, we furthermore suggest that the enzyme is located in the basolateral membrane system of the chloride cells.

Na+/K+ ATPase of mammalian origin has been reported to be amiloride sensitive (Soltoff et al. 1983; Natochin et al. 1985). Soltoff et al. added amiloride and ouabain together to the assay medium, a procedure which in our hands leads to overestimation of the amiloride effect on Na+/K+ ATPase (not shown). Furthermore, experiments with a pure Na+/K+ ATPase preparation (de Pont, pers. comm.) indicate that the dose-response curve for Na+/K+ ATPase inhibition differs markedly from the results presented in this chapter. On the other hand, at present we cannot exclude the possibility that the amiloride sensitive fraction of total Na+-ATPase activity in tilapia gills contains some Na+/K+ ATPase activity; although clear differences in K+ and NH4+ sensitivities of both enzyme activities seem to contradict such a possibility. Interestingly, amiloride did not inhibit K+-nPPase activity in our preparation (not shown). Furthermore, adaptation of tilapia to seawater or to low pH environments alters the ratio Na+/H+ ATPase versus Na+/K+ ATPase activity (Balm et al., in preparation), indicating a physiological role for the amiloride sensitive Na+-ATPase.

Our data on tilapia plasma K+ concentrations and NH4+ concentrations (see also King and Goldstein 1983) show that these values are near the $K_a$ and $K_i$ values of the Na+-ATPases we measured in this study. We therefore suggest that these ions might be important physiological factors modulating active Na+ transport, a possibility often overlooked in fish physiology. Teleost fish are ammoniotelic and produce predominantly ammonia (Smith 1929), which — at physiological pH — is ionized to NH4+ immediately after production. More than 90% of the nitrogen is excreted via the gills and current evidence indicates that under normal conditions a relatively fixed 25% of this excretion takes place via NH4+ excretion (Evans 1984b; Heisler 1984b; Evans and Cameron 1986). Fish gill Na+/K+ ATPase has been shown to be sensitive to NH4+ in vitro, its sensitivity being greater (freshwater Opsanus beta, Mallery 1979), equal (goldfish, Busacker and Chavin 1981; seawater Opsanus beta, Mallery 1983), or lower (chinook salmon, Johnson et al. 1977; sea bass, Trigari et al. 1985, and 10% seawater Opsanus beta Mallery 1983) to that for K+. Trigari et al. (1985) calculated an apparent $K_a$ for NH4+ of 5.6 mM for sea bass, a value similar to ours.

Kerstetter and Keeler (1976) concluded from their data on isolated gills of trout that Na+/NH4+ exchange was located basolaterally in the gill lamellae, while Claiborne et al. (1982) and Mallery (1983) also linked Na+/NH4+ exchange to basolateral Na+/K+ ATPase. Na+/NH4+ exchange by Na+/K+ ATPase has been shown to occur in mammalian tissues as well (Garvin et al., 1985). Our results however indicate that the amiloride sensitive Na+-ATPase might even be more important in teleost freshwater NH4+ extrusion than Na+/K+ ATPase. In fact, at the NH4+ plasma concentrations measured in tilapia plasma, this amiloride sensitive Na+-ATPase might be mediating basolateral Na+/NH4+ exchange exclusively, whereas the sensitivity of Na+/K+ ATPase to NH4+ might not be of physiological significance in tilapia gills.
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