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PROTON TRANSPORT AND Na^+/H^+ EXCHANGE IN VESICLES ISOLATED FROM SOCKEYE SALMON (*Oncorhynchus nerka*) KIDNEYS DURING MIGRATION FROM SALT TO FRESH WATER

Eric G. Holmberg and Nathan Turner

Department of Chemistry/Physics, University of Alaska Anchorage,
Anchorage, Alaska 99508

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Summary: Renal epithelial function, proton flux and sodium stimulated proton flux, was observed in vesicles isolated from the brush border of the proximal tubule of Sockeye Salmon (*Oncorhynchus nerka)* during migration. Brush border membrane vesicles (BBMV) were isolated from the body kidney of Sockeye Salmon using aggregation/differential centrifugation techniques. Vesicle purity was tested using a series of epithelial and basal lateral markers including alkaline phosphatase, maltase, gamma-glutamyl transferase (GGTP), Mg^{2+}-activated ATP-ase, $Na^+ + K^+$-activated ATPase, and 5'-nucleotidase and the lysosomal marker acid phosphatase. An enrichment/depletion factor for each marker was determined by comparison of purified BBMV with kidney homogenate. Vesicles exhibit an enrichment factor for alkaline phosphatase, GGTP, maltase, Mg^{2+}-activated ATP-ase, $Na^+ + K^+$-activated ATPase, and 5'-nucleotidase. A depletion factor was observed for acid phosphatase. Vesicle integrity was tested by measuring the time course of proton flux in the presence of a pH gradient. Amiloride sensitive sodium stimulated proton flux was observed in these vesicles. The presence of sodium caused a saturable increase in the rate of proton flux, indicating the activity of a sodium/proton antiport protein in BBMV.

The anadromous behavior, sexual maturation, and death of Pacific Salmon provide a unique opportunity to examine an organism as it undergoes a dramatic degeneration and ageing process in a relatively short period of time. We have isolated epithelial membranes from the kidney of sockeye salmon (*Oncorhynchus nerka*) to examine the effect of migration, sexual maturation, and spawning induced death on specific isolated renal epithelial functions as a model for ageing. The kidney is responsible for a variety of absorptive and secretive processes. pH regulation occurs by several different processes, included are proton secretion and Na^+/H^+ exchange. Proton transport and the electroneutral sodium/proton exchange can be measured by a variety of techniques including, and not limited to: direct electrode measurement (2), dialysis

Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N-ethane-sulfonic acid; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; BBMV, brush border membrane vesicles.

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(3), and fluorescent probes(1,4). In an attempt to examine single functions, samples from the brush border of the proximal tubule of the body kidney of sockeye salmon were prepared using existing isolation techniques (1,5). We have examined specific transport properties of the epithelial brush border of the kidneys of migrating sockeye salmon.

MATERIALS AND METHODS

Sample Collection: All fish were obtained during the 1990 migration of Sockeye Salmon (*Oncorhynchus nerka*) on the Kenai Peninsula, Alaska. Kidney samples were obtained from the mouth of the Kenai River. Salmon were bright silver, early run, and represent sexually mature salmon.

Vesicle Isolation: Renal brush border membrane vesicles were prepared by a modified method of Holmberg, et al. (1) Kidneys were trimmed by removal of the most anterior and posterior portion of the whole kidney, leaving only the body kidney. The body kidney, represents the major site of blood filtration with the presence of glomerular nephrons. (7) Concentration of vesicles and homogenate was determined by the method of Lowry (8) and Bradford (9) using trichloroacetic acid precipitation. Both vesicles and homogenate were adjusted to a concentration of approximately 30 mg/ml.

Materials: All chemicals and assay procedures were obtained from Sigma (St. Louis, MO). Ouabain sensitive Mg^{+}-ATPase was assayed according to Jorgenson and Skou. (10) All absorbance measurements were performed on a Hewlett Packard 8452 A Diode Array Spectrophotometer.

pH Jump Experiments: pH jump experiments were performed using the fluorescent probe acridine orange. Vesicles were incubated in the standard homogenization buffer at pH 6 in the presence of 6 μM acridine orange. The vesicles were introduced into identical buffer at pH 9 with and without the presence of NaCl. The results were a net proton flux from the inside of the vesicle to the exterior buffer and a final measured pH of 7.5. The fluorescence of acridine orange, excitation 493 nm/emission 530 nm, was monitored as a function of time on a Perkin-Elmer Model 650S Spectrofluorimeter. The dead time of the experiments was 3 seconds and the fluorescence was monitored for 1 minute at varying intervals. Experiments were performed in the presence of 150 mM *N*-methylglucamine (NMG)-gluconate buffer (preloaded into the vesicles and present in the exterior buffer) to negate any parallel leak pathways that may be electrically coupled. Separately, pH flux measurements were performed in the presence of K-gluconate and valinomycin to negate any electrically coupled pathways. All experiments and procedures were performed at 4°C.

Data Analysis: Data were fit to a first order rate equation of the form: $A_t=A_{inf}\ (1-e^{-kt})$ + offset using a non linear least squares fitting procedure, Grafit (11), where A_t is the fluorescence at time t, A_{inf} is the maximum fluorescence, t is the time, and k is the rate constant. The offset allows for varying degrees of background fluorescence. Kinetic data were analyzed according to the methods of Lineweaver Burk and Hill. (12,13)

RESULTS AND DISCUSSION

ENZYMATIC STUDY: Relative BBMV (brush border membrane vesicles) purity was determined by comparing the specific activity of epithelial and basal lateral markers of the

TABLE 1

ENZYME	HOMOGENATE ACTIVITY	VESICLE ACTIVITY	ENRICHMENT/(DEPLETION) FACTOR
Mg^+-activated ATPase	9.22±0.15	15.56±1.37	1.69
$(Na^+ + K^+)$-Activated ATPase	5.760±.29	12.06±0.95	2.09
Maltase	6.50±1.20	85.80±3.90	13.20
Alkaline Phosphatase	0.65±0.32	8.86±0.70	13.63
Gamma Glutamyl Transpeptidase	3.88±0.35	41.50±4.70	10.70
5'-Nucleotidase	2.00±0.28	19.01±0.44	9.50
Acid Phosphatase	0.324±0.05	0.179±0.09	(0.55)
Total Protein	32.1mg/ml	30.50mg/ml	

Activities are reported as absorbance units/sec/mg total protein (Mg^+-activated ATPase and maltase) or specific activity/mg total protein (all others). Results are reported as Means ± S.D. (n=3) for BBMV and kidney homogenate, normalized for total protein concentration, and were obtained from bright silver early run salmon.

isolated vesicles to the kidney homogenate. Table 1 shows the results of a comparison of enzymes specific for epithelial membranes, basal lateral membranes, and lysosomal markers. Alkaline phosphatase, maltase, gamma glutamyl transpeptidase, and 5'-nucleotidase can be considered as predominantly located in the proximal tubule epithelial brush border. Mg^{+2}-activated ATPase can be considered to be mitochondrial markers of the basal plasma membrane of the distal or proximal tubule cells. $(Na^+ + K^+)$-activated ATPase is a plasma membrane enzyme that is concentrated in the region of the basal infoldings of the proximal tubule cell. Acid phosphatase can be considered to be a lysosomal marker. (14,15,16)

Data indicate that there was an enrichment of all enzymatic markers with the exception of acid phosphatase in BBMV over kidney homogenate. Acid phosphatase (0.55 enrichment), a lysosomal marker, was depleted in the BBMV fraction in comparison to the kidney homogenate. Some questions seem to exist on the specific activity of 5'-nucleotidase in the presence of other enzymes such as alkaline phosphatase. (14)

There are several explanations for the slight enrichment of the basal lateral markers in the BBMV sample, this enrichment may be due to incomplete homogenization and insufficient separation of the epithelial microvilli from the basal cells. Contamination may possibly exist from the distal cells of the nephron. The differential centrifugation/Mg^+ aggregation could lead to contamination due to incomplete aggregation of the basal cell membranes and the presence of the basal cell membranes in the supernatant of the low speed spins. It has been speculated that the basal lateral cell markers may exist on the luminal membrane, to a certain extent. (16)

Acid phosphatase, a lysosomal marker, was depleted in the preparation. This result is different from those results obtained by Murer et al. (2) in rats and George and Kenny (14) in rabbits, where an enrichment, although slight, was found in the BBMV over the homogenate. Murer et al. (2) have found that particles containing acid phosphatase may have the same sedimentation properties of BBMV in their preparation and see a slight enrichment. Our data suggest that the activity is still present in the vesicle preparation but there is less lysosomal activity in these preparations.

Enzymatic data are in general agreement with previously obtained data in rabbits (5) and rats (16) with the exception of a depletion factor for acid phosphatase. Biber et al. (16) have speculated that acid phosphatase is soluble and may be present in BBMV fractions due to their similar sedimentation behavior to that of BBMV because of a possible presence in substructures or similar aggregates, a native constituency in BBMV, or an interaction between the BBMV and the protein. Our data seem to suggest that in BBMV from salmon kidneys there is a depletion of acid phosphatase in the fish at this particular stage of migration. This would seem to eliminate the possibility of a significant native constituency in the BBMV as compared to basal lateral membranes in these samples. The depletion factor, 0.55, is small and may indicate a possible weak protein/BBMV interaction in the final BBMV or a reduced presence of acid phosphatase in the basal lateral cells. Heidrich et al. have found that rat renal brush border membrane vesicles isolated by free flow electrophoresis have a depletion factor similar to our results. (18) Aggregates of particles containing acid phosphatase seem to have a different sedimentation property than that of BBMV in the fish kidney isolation procedures used for these data. BBMV isolated by these methods in salmon seem to follow an enzyme profile that would indicate that there is an enrichment of luminal markers between the original homogenate and final BBMV.

PROTON FLUX EXPERIMENTS: Figure 1 shows the fluorescent response of the pH sensitive probe acridine orange when acridine orange loaded BBMV isolated from salmon kidneys were challenged by a pH gradient. The time course was fit using a single exponential function plus an offset by non linear least squares analysis. The time course for the flux of protons from the

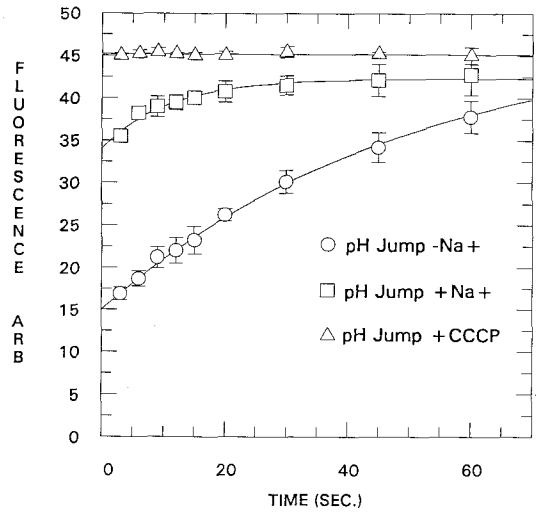

Figure 1. BBMV in 100 mM mannitol, 10 mM HEPES, 6 μM acridine orange, pH 6.0,were mixed with identical buffer, no vesicles and pH 9.0, for a final pH of 7.5. The fluorescent response of acridine orange (ex 493, em 530) was monitored as a function of time. The bottom curve (circle) represents the response of BBMV to the above described pH reequilibration, $k=0.02\pm0.0015$ sec^{-1}, amplitude 17.9 ± 1.11 arb. The middle curve (squares) represents a pH reequilibration with the addition of 500 mM NaCl to the external buffer, $k=0.09\pm0.003$ sec^{-1}, amplitude 25.72 ± 0.56 arb. The top curve (triangle) represents a pH reequilibration in the presence of the protonophore CCCP (5 μM), does not fit a single exponential function. Points represent the average of n=6 trials $\pm$ S.D.

inside of the vesicle to the exterior buffer was 0.02 ± 0.0015 sec^{-1} with an amplitude of 17.9 ± 1.11 arb. The limit indicates the relative amount of proton flux that occurred during the pH jump.

The rate of the fluorescence increase can be increased by the presence of external sodium. It is known that a Na^+/H^+ antiport pathway exist in the brush border membrane from other species, and the presence of sodium in the external buffer results in a faster rate of proton transfer during pH equilibration in vesicles isolated from the proximal tubule brush border. (1,17) Results from pH reequilibration in the presence of external sodium, indicating the presence of Na^+/H^+ antiport stimulated proton flux, were observed and measured from figure 1. The time constant for the rate of proton flux in the presence of 500 mM sodium was $0.09\pm.003$ sec^{-1} and the amplitude was 25.72 ± 0.56 arb. Experiments were controlled by the addition of the protonophore CCCP, which collapses proton gradients at a rate not observable by our instrumentation.

By varying the sodium concentration from 0 to 1000 mM and observing the rate of proton collapse in BBMV from salmon kidneys a plausible mechanism for Na^+/H^+ antiport was tested. The average initial rate of proton collapse versus various sodium concentrations showed a

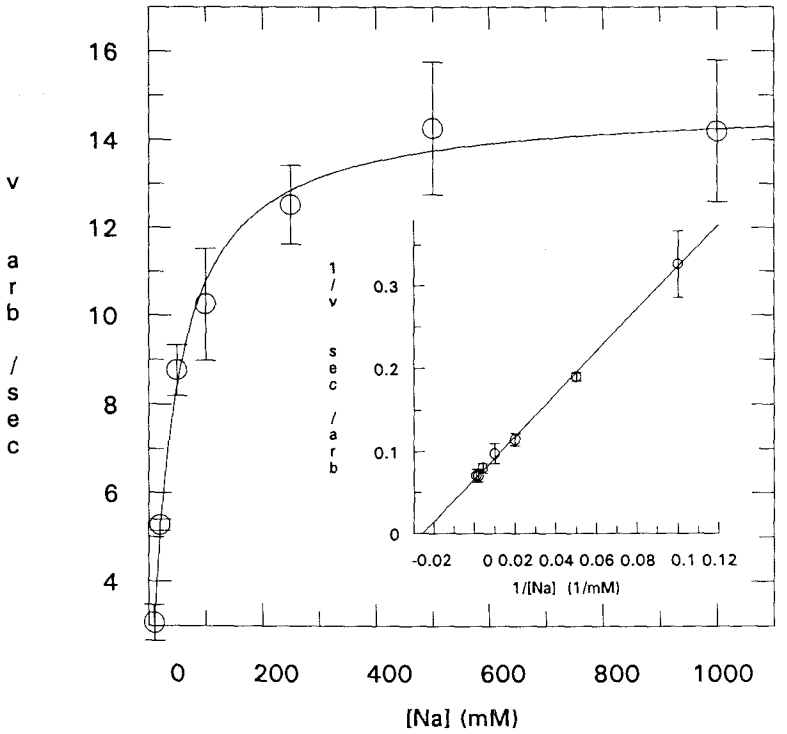

Figure 2. Average initial rates of proton equilibration (arb. fluorescence units/sec.) vs. sodium concentration (mM). Rates of proton equilibration were measured in the presence of various concentrations of sodium and show a saturable response. Inset, Lineweaver-Burke analysis (arb. fluorescence units/sec.$^{-1}$ vs. mM^{-1}) of data shows a K_m=38.21±0.769 mM and V_{max}=15.02±0.720 arb. f.u./sec. Hill analysis shows a Hill coefficient of 0.9737±0.021. Points represent the average of n=6 trials ± S.D.

saturable rate at about 15 fluorescent units/sec. on our instrumentation. (fig. 2) Lineweaver-Burk analysis (fig. 2, inset) illustrates the maximum rate of the Na^+/H^+ antiporter in the BBMV, numerical analysis yields V_{max}=15.02±0.720 fluorescent units/sec and K_m=38.21±0.769 mM. These data were analyzed according to the Hill equation to investigate the cooperativity of the Na^+/H^+ antiport mechanism. Analysis revealed that the Hill coefficient was 0.937±0.0214 for the above data. Indications are that the mechanism for sodium proton antiport follows standard kinetic behavior and is probably not cooperative. (13)

Sodium/proton transport showed an apparent inhibition in the presence of amiloride, a diuretic known to inhibit sodium stimulated transport in other epithelia. (20) Inhibition of sodium stimulated proton transport from 15% to 72% was observed as a function of amiloride concentration (10^{-4} to 10^{-3} M) from control.

Warnock et al. (4) and Kinsella et al. (19) have estimated the K_m of the sodium/proton antiport in rabbit renal BBMV to be 13.4 mM and 5 mM, respectively. Our value of 38.21 is

significantly higher than those of rabbits. Obviously, there is a great difference in the animals being studied. Possible functional explanations may include the salt conservation mechanism that the fresh-water phase of the salmon at this stage exhibits. In the fresh water the kidney acts as a water pump and a salt conserving organ. In salt water the reverse is true, salts are excreted, almost exclusively, and water is conserved. This explanation does not necessarily account for the actual salt conservation, but the higher K_m indicates a higher saturation point for this antiport protein in this particular BBMV preparation compared to others. (4,19)

This unique model is useful in ageing studies due to the short time course in which an apparently healthy normal fish undergoes a dramatic metamorphosis in which sexual maturation, spawning, and death occurs. Migrational stages are easily monitored due to the annual migration patterns that the fish follow. (6) Brush border membrane vesicles isolated from the kidneys could allow detailed investigation of the apparent breakdown of the renal reabsorption and secretion function. Comparison of enzyme profiles, proton and sodium/proton equilibration, and water and non-electrolyte transport in fish at different stages of migration are presently being investigated.

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