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Stoichiometry of a Cl^--translocating ATPase

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It has been determined that Mg^{2+} stimulates phosphorylation while Cl^- stimulates dephosphorylation of the chloride pump during its reaction sequence. The stoichiometry of ATP hydrolyzed to Cl^- transported during a single cycle of the reaction sequence was ascertained. Intracellular concentrations of ATP, ADP and inorganic phosphate were determined and, coupled with an estimate of the standard free energy of hydrolysis for ATP, the operant free energy for ATP hydrolysis was calculated. Because the operating free energy of the pump is approximately one-half the energy obtained from the total free energy of ATP hydrolysis, the only possible integral stoichiometries are one or, at the most, two net charges transported per cycle per ATP hydrolyzed.

Aplysia; Cl^--pump; Stoichiometry; ATPase; Reaction sequence

1. INTRODUCTION

Anion-stimulated ATPase activity was first described in frog gastric mucosa [1] where it was thought to play a role in the simultaneous transport of H^+, HCO_3^-, and Cl^-. Thereafter, discovery of anion-stimulated ATPase was demonstrated in many mammalian tissues [2,3] and some lower vertebrates such as: *Necturus* [4], eel [5], trout [6], and goldfish [7]. With the exception of some recent studies [8,9], very little information is currently available on anion-stimulated ATPase activity in any biological tissue.

Transepithelial Cl^- flux studies [10,11] in *Aplysia californica* gut have shown that the short-circuit current is carried by a net active Cl^- absorptive flux. It was hypothesized that Cl^- absorption across the *Aplysia* gut is mediated by a primary active transport process [12,13] located in the basolateral membrane (BLM) [13]. Lending credence to this idea were the demonstrations of: (1) the existence of Cl^--stimulated ATPase activity in *Aplysia* foregut absorptive cell BLMs [14], and (2) that there was an electrogenic ATP-dependent Cl^- uptake in these same *Aplysia* foregut absorptive cell BLM vesicles [15,16]. Furthermore, both Cl^--stimulated ATPase and ATP-dependent Cl^- transport activities were reconstituted into proteoliposomes [17]. Therefore, the present study was undertaken to assess the stoichiometry of ATP hydrolyzed to Cl^- transported by the ATPase in order to further characterize properties of the Cl^- pump.

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2. MATERIALS AND METHODS

Seahares (*Aplysia californica*) were obtained from Marinus Inc. (Long Beach, CA) and were maintained at 25°C in circulating filtered seawater. Adult *Aplysia* (400–600 g) were used in these experiments and in most cases only animals that had been kept in the laboratory under the above conditions for ≤ 1 week were used. Posterior foregut (crop) was used as in all other studies [13,17]. The animals were sacrificed and their guts were removed, slit longitudinally, and rinsed off in seawater medium. The gut was then positioned between two halves of a Lucite chamber described previously [12,18], which allowed measurement of transepithelial electrical potential (ψ_{ms}) and, simultaneously, the introduction of microelectrodes into the surface epithelial cells. The chamber exposed 22.9 mm^2 of tissue to oxygenated seawater medium. The mucosal chamber volume was 4.5 ml; the serosal chamber volume was 8.5 ml. Both compartments were perfused at a rate of approximately 1 ml/min with fresh medium.

The formulae for the seawater media were (in mM): TrisCl or NaCl, 462.0; $MgSO_4 \cdot 7H_2O$, 2.4; KCl, 10.0; $KHCO_3$, 2.4; $MgCl_2$, 9.8; $CaCl_2$, 11.4. The total osmolality of the bathing media was 1,000 mosmol/l and the final pH was 7.8.

Single-barreled chloride-selective liquid ion-exchanger microelectrodes were constructed as described by Walker [19]. Briefly, clear Pyrex capillary tubing was pulled with Kopf model 700 C pipette puller (DKI Instruments) to a tip diameter of less than 0.5 μm and dipped in 1% Siliclad (Clay Adams) in 1-chloronaphthalene until approximately 250 μm of the tip was filled. These were heated to 235°C for 1 h. On cooling, the siliconized microelectrodes were allowed to take up chloride exchanger (Corning Glass Works) until 250 μm of the tip was filled. The space above the exchanger was filled with 500 mM KCl. The chloride-selective microelectrodes were calibrated after impalement of the absorptive cell in solutions of 5, 50, and 500 mM NaCl. The chloride activity of these solutions (a^o_{Cl}) was taken to be 4.64, 41.1, and 340 mM as calculated from the Debye–Hückel equation [19]. The slope of the electrode response to changes in chloride activity (a_{Cl}) was linear and averaged 54.0 mV per decalog change in a_{Cl} in these same electrodes. The selectivity of this exchanger was determined and its selectivity of Cl^- over $H_2PO_4^-$ was 64:1, whereas over HCO_3^- it was 11:1.

Microelectrodes for measurements of membrane potential were pulled to the same dimensions as the chloride-sensitive microelectrodes and filled with 1 M potassium citrate as described by Tasaki

et al. [20]. Tip resistance of these open-barreled microelectrodes ranged between 6 and 30 MΩ. Recordings of mucosal membrane potential (ψ_m) were considered acceptable if (1) the tip potential of the microelectrode was not greater than 5 mV and did not change by more than 3 mV between penetration and withdrawal; (2) the membrane potential repolarized again in those cases where it had begun to decline toward zero; (3) the membrane potential did not change more than ±1 mV for at least 30 s.

Chloride-specific and membrane potential-sensing microelectrodes were connected via Ag/AgCl half-cells to either a Keithley model 616 digital electrometer (Cl-specific) or a WPI model M701 amplifier (membrane potential-sensing electrode). The output of the latter electrode was read with a Digitec model 268 digital millivoltmeter. These voltages were, in turn, recorded on a Brush model 220 recorder. The transepithelial potential (ψ_{ms}) was monitored by an additional voltmeter and recorded on a second channel of the Brush recorder. The ψ_{ms} was measured with calomel half-cells that made contact with the chamber through saturated KCl-agar bridges. All potentials were referenced to the mucosal solution. The mucosal bridge was used as a reference electrode for calibration of the chloride-specific microelectrodes.

The experimental protocol for single-barreled measurements was as follows: after the excised tissue was placed in the chamber, microelectrodes were advanced into cells lining the gut villi to obtain an independent estimate of ψ_m, after which, chloride-selective microelectrodes were passed into the villus epithelial cells. After one to four successful recordings the chloride microelectrode was calibrated in solutions described previously. The difference between the mean of ψ_m and the mean potential observed with the chloride-selective microelectrodes was equated with intracellular chloride activity. The intracellular chloride activity (a^i_{Cl}) was calculated using Eqn. 1 where ψ^i is the potential of the chloride electrode in the cell

$$a^i_{Cl} = a''_{Cl}/e^{2.303[\psi^i - \psi_m) - \psi'']/S} \quad (1)$$

and a''_{Cl} is the activity of chloride, ψ'' the potential of the chloride electrode in 500 mM NaCl or TrisCl, and ψ_m is the mean mucosal membrane potential [21]. S is the slope of the electrode response and is calculated as $S = \psi'' - \psi' / \log (a''_i/a'_i)$ where ψ^i and a_i are the potential of the electrode and the chloride activity of the solution in 5 or 50 mM NaCl or TrisCl.

All microelectrodes (membrane potential-sensing and ion-selective) were advanced with a Kopf model 607 W hydraulic microdrive after the microelectrode was aligned to within about 200 μm of the tip of the villus under × 60–100 magnification. All experiments were conducted at room temperature in a shielded cage resting on a cement table, thus reducing electrical and mechanical interference.

Preincubation (30 min) and incubation of scraped mucosal cells at 25°C for 30 min were done in the same NaCl and TrisCl seawater media, respectively, described previously (vide supra) for the determinations of intracellular ATP, ADP and inorganic phosphate (P_i) concentrations. Specifically, this followed the method of White [18] where segments of gut were cut open and pinned to paraffin in the bottom of a Lucite chamber containing continuously oxygenated seawater. With a dissecting microscope and fine scissors, strips of villous tissue were cut from the gut and these were scraped for mucosal cells. After incubation, all remaining experimental groups of cells were frozen in liquid nitrogen until the analyses for nucleotides were undertaken. Extracts of tissue were prepared and ATP analyses made as described by Lamprecht and Trautschold [22]. ADP was determined as described by Adam [23]. P_i was determined as described by Fiske and SubbaRow [24].

The data obtained were analyzed statistically by Student's t-test.

Table I

Mucosal membrane potentials (ψ_m), transepithelial potentials (ψ_{ms}) and calculated serosal membrane potentials (ψ_s) in NaCl and TrisCl seawater media

	ψ_{ms} (mV)	ψ_s (mV)	ψ_{ms} (mV)	n
NaCl	−61.9 ± 1.2 (31)	−60.0 ± 2.0 (31)	−1.9 ± 0.5 (31)	6
TrisCl	−66.2 ± 1.5 (38)	−63.1 ± 1.9 (39)	−3.1 ± 0.6 (40)	6
	$P < 0.05$	$P < 0.05$	$P < 0.05$	

Values are means ± S.E. Numbers in parentheses are numbers of observations; n, number of animals. Polarities of ψ_m and ψ_{ms} are relative to mucosal solution. Polarity of calculated ψ_s is relative to cytoplasm.

3. RESULTS

The first series of experiments examined ψ_m, ψ_{ms} and the potential difference across the basolateral membrane (ψ_s) of *Aplysia* foregut absorptive cells. ψ_s is a calculated value that is determined by the algebraic summation of ψ_m and ψ_{ms} from the equation: $\psi_m + \psi_s = \psi_{ms}$. As can be seen in Table I, there was a hyperpolarization of both ψ_m and ψ_{ms} when Na^+ was replaced in the seawater bathing medium with $Tris^+$. This also causes a hyperpolarization of the calculated value of ψ_s.

The next series of experiments was designed to determine the free ion concentration or activity of Cl^- (a^i_{Cl}) in *Aplysia* villus foregut absorptive cells. Table II shows that there is a small but significant decrease in a^i_{Cl} when Na^+ is replaced in the extracellular seawater medium with $Tris^+$. As in the previous set of data, ψ_m hyperpolarized under these experimental conditions. However, a^i_{Cl} was always less than that predicted for electrochemical equilibrium across the mucosal membrane whether the tissue was bathed in a seawater medium containing Na^+ or $Tris^+$.

The final series of experiments was designed to determine intracellular concentrations of ATP, ADP and P_i in the *Aplysia* villus foregut absorptive cells when Na^+ is absent from the seawater bathing medium. Table III shows that [ATP] significantly decreased while [ADP] and [P_i] increased, though not significantly, when Na^+ was replaced by $Tris^+$ in the extracellular seawater bathing medium.

4. DISCUSSION

It had been previously demonstrated that the isolated gut of *Aplysia californica*, bathed in a Na^+-free seawater bathing medium, generated a serosa-negative transepithelial potential difference [11]. The short-circuit current (I_{sc}) across this preparation was totally accounted for by an active absorptive flux of Cl^-. This was in contrast to flux studies in the same preparations, which were bathed in a Na^+-containing seawater medium, that demonstrated the I_{sc} to be accounted for by active fluxes of both Na^+ and Cl^- [10]. Therefore, in the absence of Na^+, only one pump mechanism (i.e. Cl^-) is expressed in the *Aplysia californica* gut; therefore, the rationale for

Table II

Intracellular chloride activities in NaCl and TrisCl seawater media

	a^{i}_{Cl} (m)	a^{eq}_{Cl} (m)	ψ_m (mV)	n
NaCl	14.3 ± 0.7 (22)	27.9	−66.3 ± 1.3 (35)	8
TrisCl	10.2 ± 0.3 (25)	23.7	−69.6 ± 1.9 (32)	8
	$P < 0.05$		$P < 0.05$	

Values are means ± S.E. Numbers in parentheses are numbers of observations; n, number of animals. Polarity of ψ_m is relative to mucosal solution. a^{eq}_{Cl} is calculated according to electrochemical equilibrium.

using a Na^+-free bathing medium to ascertain the energetic equivalents for both ATP and the Cl^--pump. It had been demonstrated by thermodynamic means that intracellular Cl^- exists below electrochemical equilibrium values [12], negating the postulation for the existence of a Cl^- active transport system in the mucosal membrane of *Aplysia* foregut absorptive cells because Cl^- movement from the luminal medium into the cytoplasm of these cells could utilize the energy implicit within its own downhill-directed electrochemical potential gradient. However, the transit of Cl^- across the BLM from the cellular cytoplasm into the serosal solution involves moving up a large electrochemical gradient for Cl^- [12]. Thermodynamically, therefore, the site for the active Cl^- transport mechanism had to be the BLM of the *Aplysia* foregut absorptive cell [12,13]. Furthermore, it was demonstrated that the BLM of *Aplysia* foregut absorptive cells contained both aspects of a Cl^- pump mechanism and, that being, Cl^--stimulated ATPase activity [14], and ATP-dependent Cl^- accumulative transport [15,16]. The transport component in BLM vesicles was further shown to be electrogenic by both indirect (ionophoretic) and direct (lipophilic cation distribution) [16] means. This rationalized the electrophysiologic examination of these cells for delineating operant free energy of the pump mechanism. Furthermore, both Cl^--stimulated ATPase and ATP-dependent Cl^--transport activities were reconstituted into preformed liposomes [17,25], and the reaction sequence of the Cl^- pump was ascertained [25]. Mg^{2+} was shown to phosphorylate the ATPase, while Cl^- stimulated dephosphorylation with its simultaneous transfer from the cytoplasmic side of the BLM to the extracellular side during the reaction sequence [25]. However, due to methodological differences between the two pump assays (Cl^--ATPase activity [14,25] and ATP-dependent Cl^- transport [16,25]) and a significant proportion of Cl^- binding to plasma membrane fractions [16] during the transport assay, the stoichiometry of ATP hydrolyzed to Cl^- transported could not be validly derived. Therefore, the present study was undertaken to assess the stoichiometry of ATP hydrolyzed to Cl^- transported by the ATPase in order to further characterize properties of the Cl^- pump. Therefore, it was thought to be justifiable to use a thermodynamic, energetic approach in order to decipher the stoichiometry of ATP hydrolyzed to Cl^- transported per reaction cycle of the ATPase. This was similar to that employed by Slayman and his colleagues for fungal H^+-ATPase [26] and Gradmann and his colleagues for algae Cl^--ATPase [27] in ascertaining the stoichiometry of these transport ATPases.

Table III

Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and inorganic phosphate (P_i) intracellular concentrations in NaCl and TrisCl seawater media

	[ATP] (mM)	[ADP] (mM)	$[P_i]$ (mM)	n
NaCl	3.00 ± 0.26 (16)	1.10 ± 0.19 (16)	12.5 ± 2.0 (16)	6
TrisCl	2.57 ± 0.21 (22)	1.16 ± 0.10 (22)	13.0 ± 2.1 (22)	6
	$P < 0.05$	N.S.	N.S.	

Values are means ± S.E. N.S., not significant. Numbers in parentheses are numbers of observations; n, number of animals.

The total energy consumed by the Cl^- pump (in the absence of Na^+) is defined by its electromotive force, which is reflected in the differences between cytoplasmic versus extracellular Cl^- activities and the electrical potential across the BLM ($\Delta\psi_s$). Utilizing the data in both Tables I and II, the operating free energy of the Cl^- pump can be calculated. Since there is a 34.0-fold difference in extracellular versus intracellular Cl^- activity and the $\Delta\psi_s$ is −63 mV (cytoplasm negative), with an orientation opposite to that of the chemical potential, the total or operating free energy utilized by the pump mechanism is approximately 140 mV.

To calculate the free energy of hydrolysis for ATP, the following equation is used:

$$\Delta G_T = \Delta G_{obs} + RT\ln\frac{[ADP]_i[P_i]_i}{[ATP]_i} \quad (2)$$

where ΔG_T = total free energy of ATP hydrolysis; ΔG_{obs} = standard empirical free energy of ATP hydrolysis; R = Boltzmann gas constant; T = absolute temperature; $[ATP]_i$, $[ADP]_i$, $[P_i]_i$ = intracellular concentrations of ATP, ADP and P_i, respectively. By linear interpolation from the tabulated results of Phillips et al. [28] and calculations according to Alberty [29], it is possible to estimate a standard empirical free energy (ΔG_{obs}) for the hydrolysis of ATP equal to 5.56 kcal/mol with the following parameters specified: ionic strength = 0.5, intracellular pH = 7.5 [14,28,29], and magnesium concentration = 5 mM [14,25,28,29]. The data for calculation of ΔG_T (Eqn 2) are listed in Table III. Assuming the reactants to be homogenously distributed, the free energy available to the electrogenic Cl^- pump (in the absence of Na^+ pump activity) is −(5.56 + 0.74) = −6.30 kcal/mol, which corresponds to 270 mV.

In summary, the electromotive force of the Cl^- pump,

which is defined as its operating free energy, approximates 140 mV, while the total energy derived from ATP hydrolysis, under Na^+-free conditions, is estimated at 270 mV. A two-charge process generating 140 mV would require a free energy equivalent to 280 mV, equal within limits of precision of the overall calculation to the maximal free energy available from ATP hydrolysis (i.e. 270 mV). The only possible integral stoichiometry, under these Na^+-free experimental conditions, is two net charges of Cl^- per ATP hydrolyzed per reaction cycle. Of course, this does not, in any way, negate the possibility of one net charge of Cl^- per ATP hydrolyzed per reaction cycle with the additional free energy utilized for other metabolic purposes. However, a $1Cl^-/1ATP$ stoichiometry implies that only cells in an energy-rich environment could afford this less-efficient pump stoichiometry. This is probably not the case with the seaborne *Aplysia*. Physiologically, the electrogenic Cl^- pump [9,16], most likely, transports two Cl^- per ATP hydrolyzed, if indeed, the greater pump electrogenicity created with a 2:1 versus a 1:1 stoichiometry is utilized to generate a significantly larger electrochemical driving force. This larger electrochemical driving force created by a 2:1 versus a 1:1 Cl^-/ATP stoichiometry could fuel secondary, electrophoretic (or electroneutral) transport processes such as the nutritional uptake of sugars and/or amino acids [9]. The 2 Cl^-/ATP stoichiometry corresponds exactly to the stoichiometry of 2 Cl^- transported/−ATP hydrolyzed derived for *Acetabularia* Cl^- pumps [9,27] utilizing similar electrophysiological methods in ascertaining the data. Slayman et al. [26] have also used these electrophysiological approaches in defining the stoichiometry of fungal H^+-ATPases, which, incidentally, for the electrogenic H^+ pump was 2 H^+ transported per ATP hydrolyzed per reaction cycle.

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REFERENCES

[1] Kasbekar, D.H. and Durbin, R.P. (1965) Biochim. Biophys. Acta 105, 472–482.
[2] Schuurmans Stekhoven, F. and Bonting, S.L. (1981) Physiol. Rev. 61, 1–76.
[3] Gerencser, G.A. and Lee, S.H. (1983) J. Exp. Biol. 106, 143–161.
[4] Wiebelhaus, V.D., Sung, C.P., Helander, H.F., Shah, G., Blum, A.L. and Sachs, G. (1971) Biochim. Biophys. Acta 241, 49–56.
[5] Morisawa, M. and Utida, S. (1976) Biochim. Biophys. Acta 445, 463.
[6] Bornancin, M., DeRenzis, G. and Naon, R. (1980) Am. J. Physiol. 238, R251-R259.
[7] DeRenzis, G. (1971) J. Exp. Biol. 63, 587–602.
[8] Gerencser, G.A. (1986) Trends Life Sci. 1, 1–18.
[9] Gerencser, G.A., White, J.F., Gradmann, D. and Bonting, S.L. (1988) Am. J. Physiol. 255, R677–R692.
[10] Gerencser, G.A. (1981) Am. J. Physiol. 240, R61–R69.
[11] Gerencser, G.A. (1984) Biochim. Biophys. Acta 775, 389–394.
[12] Gerencser, G.A. and White, J.F. (1980) Am. J. Physiol. 239, R445–R449.
[13] Gerencser, G.A. (1983) Am. J. Physiol. 244, R143–R149.
[14] Gerencser, G.A. and Lee, S.H. (1985) Am. J. Physiol. 248, R241–R248.
[15] Gerencser, G.A. and Lee, S.H. (1985) Biochim. Biophys. Acta 816, 415–417.
[16] Gerencser, G.A. (1988) Am. J. Physiol. 254, R127–R133.
[17] Gerencser, G.A. (1990) Biochim. Biophys. Acta 1030, 301–303.
[18] White, J.F. (1976) Am. J. Physiol. 231, 1214–1219.
[19] Walker, J.L., Jr. (1971) Anal. Chem. 43, 89–93.
[20] Tasaki, I., Polly, E.H. and Orrego, F. (1954) J. Neurophysiol. 17, 454–474.
[21] White, J.F. (1977) Am. J. Physiol. 232, E553–E559.
[22] Lamprecht, W. and Trautschold I. (1963) in: Methods of Enzymatic Analysis, 2nd Edn., pp. 543–551, Academic Press, New York.
[23] Adam, H. (1963) in: Methods of Enzymatic Analysis, 2nd Edn., pp. 573–577, Academic Press, New York.
[24] Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400.
[25] Gerencser, G.A. and Zelezna, B. (1993) Proc. Nat. Acad Sci. USA, in press.
[26] Slayman, C.L., Long, W.S. and Lu, C.Y.-H. (1973) J. Membr. Biol. 14, 305–338.
[27] Goldfarb, V., Sanders, D. and Gradmann, D. (1984) J. Exp. Bot. 35, 626–644.
[28] Phillips, R.C., George, P. and Rutman, R.J. (1969) J. Biol. Chem. 244(12), 3330–3342.
[29] Alberty, R.A. (1969) J. Biol. Chem. 244(12), 3290–3302.