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Comparative Analysis of Mg-Dependent and Mg-Independent HCO_3^- ATPases

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Abstract The comparative analysis between two enzymes, Mg-dependent and Mg-independent HCO_3^- ATPases, were studied in synaptosomal and microsomal membrane fractions of albino rat brain, using the method of kinetic analysis of the multi-sited enzyme systems. Therefore, it can be inferred that Mg-dependent HCO_3^- ATPase belongs to the group of "P-type" transporting ATPases. Mg-independent HCO_3^- ATPase with its kinetic properties may be attributed to the group of "Ecto" ATPases.

Keywords Mg-dependent and Mg-independent HCO_3^- ATPases · P-type ATPase · Ecto-ATPase · Enzyme kinetic · Multi-sited enzyme system

Introduction

HCO_3^- ATPase is known as Mg-dependent enzyme system activated by the HCO_3^- ions ($MgHCO_3^-$ ATPase, E.C.3.6.1.3.). Mg-dependent HCO_3^- ATPase is found in the plasma membrane of animal and plant cells (Ivashenko 1977; Izutsu and Siegel 1980). The participation of this enzyme in the process of active transport of bicarbonate ion through the membrane is supposed (Simon and Thomas 1972; Gassnez and Komnick 1981; Tsakadze and Koshoridze 1976).

The asymmetric distribution of anions (mainly HCO_3^- and Cl^-) in the membrane (out >> in) accounts for their passive transport down the concentration gradient whose reverse system appears to be the existence of active transport mechanism. Transport ATPases are known to have a particularly important role in the cell functioning. Providing the asymmetric arrangement of ions in the membrane at the expense of ATP hydrolysis, they represent a complex biological machine.

But the ATPase system accomplishing ATP hydrolysis without Mg ion (Mg^{++}-independent HCO_3^- ATPase) has been observed in synaptosomal and microsomal membrane fractions of the albino rat brain (Leladze et al. 2010).

There is no available information on Mg-independent HCO_3^- ATPase in the literature. According to our data, the activation of ATPase reaction fixed by HCO_3^- ions without the participation of Mg ions should not be attributed to the so-called P-type transport ATPases system (Dzneladze et al. 2012). Thus, the place of Mg-independent HCO_3^- ATPase is unclear in modern classification.

The goal of our work was to reveal the mechanism of action of Mg-dependent and Mg-independent HCO_3^- ATPase using the method of kinetic analysis of the multi-sited enzyme systems (Kometiani 2007), as well as to perform their comparative analysis and to establish a place of each of them in ATPase classification.

Materials and Methods

The synaptosomal and microsomal membrane fractions obtained from albino rat brains via the method of differential centrifugation in a sucrose gradient [1.2–0.9 M sucrose] (Kometiani et al. 1984) was an enzymatically active material. ATPase activity (V) was assessed by the amount of isolated inorganic phosphorus (presumed to originate from ATP dissociation) per mg protein per hour. Protein concentration was measured by the Lowry method (Lowry et al. 1951). Inorganic phosphorus was measured using a modified method of (Kazanova and Maslova 1984).

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HCO_3^- ATPase was determined by the activity difference in medium with and without HCO_3^- ions. The incubation medium contained 30 mM Tris–HCl buffer; 2 mM ATP; 2 mM $MgCl_2$; 0.2 mM ouabain; and 0.4 mM EGTA.

Method for Kinetic Analysis of Multi-Sited Enzyme Systems

The method of kinetic analysis of multisite enzyme systems was applied for the analysis of the experimental curves [Kometiani 2007]. To analyze the initial velocity of an enzymatic reaction, it is required to obtain $V = f([HCO_3^-], [Mg\text{-}ATP], [Mg_f^{2+}] [ATP_f])$, (the subscript "*f*" denotes, free'), as a function of one variable, while keeping the values of other quantities constant. Therefore, in any particular experiment, the concentrations of the ligands were chosen in such a way that the enzyme reaction velocity was actually represented by one variable ligand concentration function; that is, in each experiment, the concentration of three ligands were kept constant. Then the conditions of the reaction do not vary, the enzyme functional unit structure is in a steady state, and the initial velocity is one-variable function described by the following analytical formula:

$$V = e_0 \frac{x^n \sum_{i=0}^{p} \alpha_i x^i}{\sum_{i=0}^{S} \beta_i x^i}; \quad (1)$$

where *i*-indices of the ligand (essential activator, partial effect modifier or full inhibitor) with

$$S = n + m + p \quad (2)$$

and αi and βi are the products of individual velocity coefficient and steady-state ligand concentrations; x is the variable ligand concentration; and e_o is the overall enzyme concentration. *n, m*, and *p* are power parameters and are positive integers: *n* is the number of sites for essential activators, *m* is the number of sites for full effect inhibitors, and *p* is the number of sites for partial effect modifiers. Numerical values for the parameters *n, m*, and *p* were computed using the method of kinetic analysis of the multisited enzyme systems (Kometiani 2007).

Results

Mg^{2+}-Dependent HCO_3^- ATPase

Mg-dependent ATPase activity dependence upon the concentration of HCO_3^- ions in the fraction of synaptic membranes is given in Fig. 1. By means of the analysis of

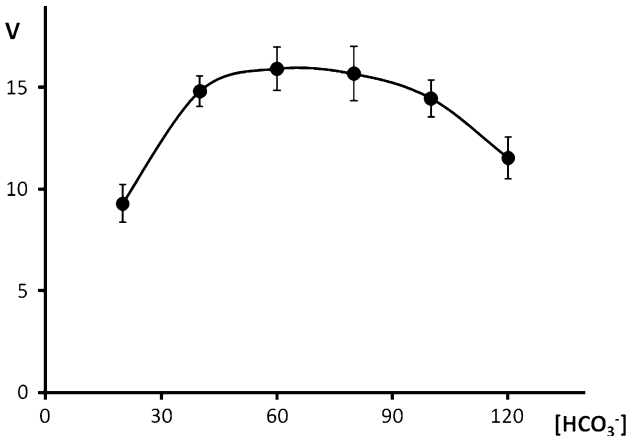

Fig. 1 HCO_3^- anion-dependent, Mg^{2+}-activated hydrolysis activity (v) on HCO_3^- ion concentration. Protein-0.01 mg/ml, ATP-1.8 mM, $MgCl_2$ = 1.8 mM, Tris-Malate = 30 mM, Ouabain = 0.2 mM

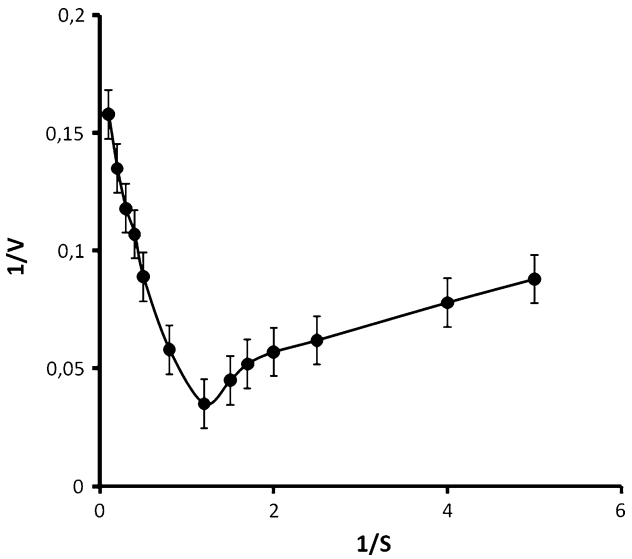

Fig. 2 Double inverse plot of the Dependence of HCO_3^- ATPase (in μmol P_i/h per mg protein) upon substrate (S = Mg-ATP) concentration (in mM), when [Mg] = [ATP]

curve geometric shape, a bell-like shape is clearly seen depending upon the HCO_3^- ion concentration via ascending and descending phases. This geometric shape is characteristic of all the transport ATPases (Tr) and appears to be a necessary, but not sufficient condition for the ligand transportation.

The study of HCO_3^- ATPase activity dependence upon the substrate concentration (S = Mg-ATP complex) in double inverse coordinates shows the following (Fig. 2): a curve has a complex geometric shape: at a high value of the argument, the enzyme activation and function rectilinearity are observed, $1/V = f(1/S)$ function has an asymptote. It is

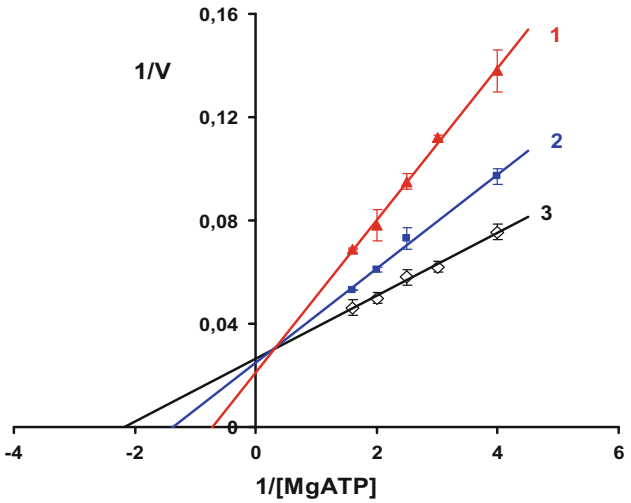

Fig. 3 Double inverse plot of the Dependence of HCO_3^- ATPase activity upon substrate concentration at different fixed HCO_3^- concentrations: *line 1* 25 mM; *2* 50 mm; and *3* 100 mM

a sufficient condition to say that the number for essential activator site for Mg-ATP is 1, $n = 1$. At low value of argument, the enzyme system is inhibited. While at mean value, it has one turning and one inflection point.

The dependence of HCO_3^- ATPase activity on the Mg-ATP low concentration in double inverse coordinates at different fixed concentrations of HCO_3 ions is shown in Fig. 3. By increasing HCO_3 ion concentration, gradient of the lines decreases, and they are intersecting in the first quarter.

The study of the dependence of Mg-dependent HCO_3^- ATPase activity on the concentration of HCO_3^- has shown that the following kinetic evidences are performed – $V = f(HCO_3^-)$ function has a bell-like shape (Fig. 1). According to this picture Mg-dependent HCO_3^- ATPase performs HCO_3^- transport. Mg-ATP appears to be a true substrate (Fig. 2). HCO_3^- ions are the activator of the enzymatic system (Fig. 3).

Mg^{++}-Independent HCO_3^- ATPase

In the process of Mg^{++}-independent HCO_3^- ATPase study, the activation of ATPase reaction without Mg ions was fixed. The reaction takes place in various membrane fractions of the brain (synapses, microsomes). As the hydrolysis of ATP occurs without Mg^{++}, the above-mentioned system is called Mg^{++}-independent HCO_3^- ATPase. The mechanism of its action was studied.

The dependence of HCO_3^- ATPase activity on the concentration of [HCO_3^-]ions (X), fixed at different values of ATP is given in Fig. 4. In conditions of $X < 100$ mM, $V = f(x)$ increases and passes to the plateau (a classical hyperbola) (Fig. 4a). But at $x > 100$ mM concentration following the plateau, there is a dramatic rise of activity and dependence is authentically rectilinear (Fig. 4b), which is kinetically unjustified. The analysis of $v = f(x)$ function at a high concentration of the argument suggests the artifact nature of HCO_3^- ion action. The function does not have bell-like shape. In this way, the transport function of HCO_3^- ATPase renders doubtful. In case of active transport, at high concentrations, HCO_3^- induced activation must be followed by an inhibitory phase.

The existence of Mg^{++}-independent HCO_3^- induced by ATP hydrolysis indicates that as distinct from Mg^{++}-dependent ATPases, free ATP but not Mg-ATP complex must be a substrate for HCO_3^- ATPase. Therefore, we have taken this into account during further study of the molecular mechanism.

Figure 5 shows the dependence of HCO_3^- ATPase activity on HCO_3^--ion concentration (X) at different fixed concentrations of ATP in double inverse values. By the calculation of regression coefficient and defining the point of interception of lines, it is possible to ascertain the character of action of enzyme system modifier (ATP in this case). If we analyze the regression coefficients (a, b, a/b) presented in the Table, we shall be convinced that for this case, −a/b parameter is authentically similar at different fixed concentrations of ATP, i.e., the interception of linear functions occurs in one point of abscissa axis. And this indicates that the enzyme affinity for HCO_3^--ions does not alter at ATP concentration changes.

The dependence of HCO_3^- ATPase activity (inverse value $1/V$) on ATP concentration has a complex character at different fixed concentrations of HCO_3^- ions (75, 100 mM) (Fig. 6). At low value of ATP [ATP] < 0.8 mM, the activation of the reaction occurs, but at high value [ATP] > 0.8 mM, the activation passes into inhibition. There is a turning point and increase of ATP concentration, which causes the inhibition of the reaction under conditions of fixed concentration of HCO_3^- ions. The inhibition induced by the increase of ATP concentration is a substrate inhibition of enzyme reaction. By calculating the regression coefficients, it emerged that at different fixed concentrations of HCO_3^- ions, b-value was identical, i.e. the curves are parallel. This means that slope does not change, but the points of intersect of the abscissa and ordinate axis do. As a result, at high ATP concentration ([ATP] > 1 mM), HCO_3^- via activation alters the catalytic constant and does not affect the specific one. For accurate analysis of curve geometric shape, the substrate dependence (an activation phase) is represented by double inverse values ($1/V = f(1/S)$ at different fixed concentrations of HCO_3^--ions (50, 75, 100 mM) (Fig. 7). As seen in the Fig. 1, there is no authentic difference in –a/b $P < 0.005$ values at different concentrations of HCO_3^--

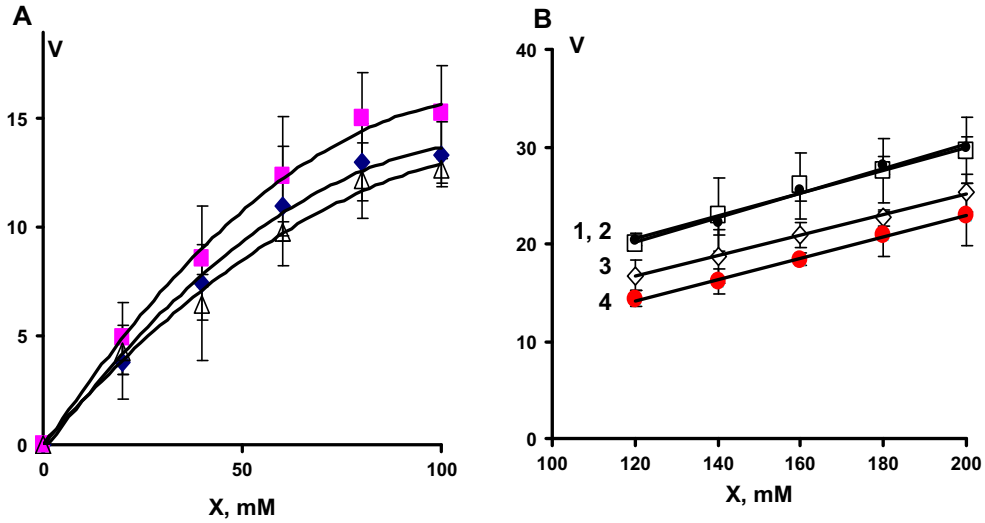

Fig. 4 Dependence of HCO_3^- ATPase activity on HCO_3^- concentration (X) at different fixed values of ATP: *1* and *2* 0.5–1 mM, *3* 1.5 mM, *4* 2 mM; **a** x < 100 mM; **b** x > 100 mM

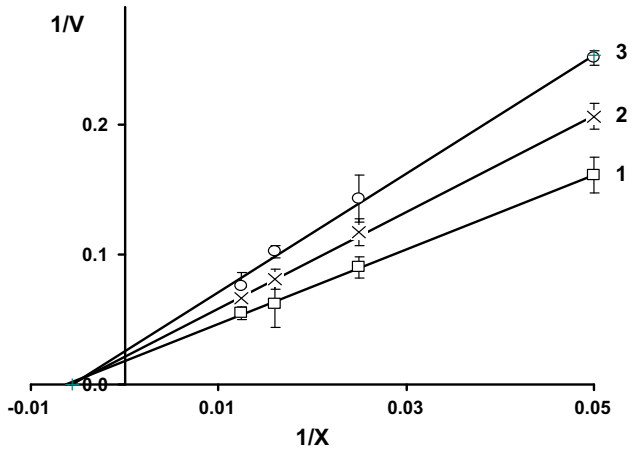

Fig. 5 Dependence of HCO_3^- ATPase activity on HCO_3^- concentration (X) in double inverse values at different fixed values of ATP: *1* 0.5 mM; *2* 1 mM; *3* 1.5 mM

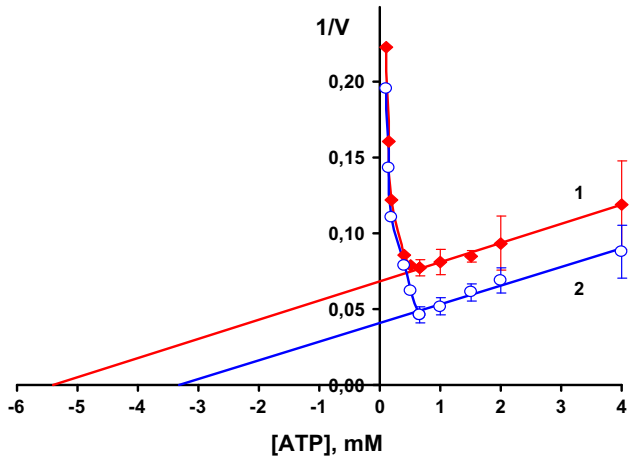

Fig. 6 Dependence of HCO_3^- ATPase activity on ATP concentration in double inverse values at different fixed values of HCO_3 ions: *1* 50 mM; *2* 100 mM

ions and straight lines intercept in one point across abscissa axis. HCO_3^- ATPase affinity for ATP remains unchanged with the rise in HCO_3 concentration, while the values of slope (b) and intercept (a) on the ordinate decline. From this, it may follow that the modifier (HCO_3^- in this case) is an activator within the given concentration ranges.

The kinetic study of Mg-independent ATPase, in particular the dependence enzyme activity upon the concentration of HCO_3^- ions ($V = f\,[HCO_3^-]$) by means of the analysis of a curve geometric shape has shown that the curve has three phase: within the concentration frames of HCO_3^- (10–100 mM), the activation of the system takes place (Fig. 4a). While increasing the concentration (up to 120 mM), an insignificant plateau is noted and by the further increase in concentration, the activation is again observed instead of the anticipated inhibition (Fig. 4b). This result casts doubt on the transport function of the enzyme. According to the data of literature, there are so-called "Ecto" ATPases, functioning from the inner side of plasma membrane, which do not need the stimulation by Mg ions as compared to transport ATPases and are inhibited by specific inhibitors-ARL67156 (6-*N*,*N*-Diethyl-β-γ-dibromomethylene-D-adenosine-5′-triphosphate trisodium salt hydrate, FPL 67156) (Drakulich et al. 2004; Levesque et al. 2007).

The effect of the inhibitor on HCO_3^- ATPases of both types was studied. When the substrate [Mg-ATP] appears to be a complex, then the introduction of ARL into the incubation medium does not change the activity of HCO_3^- ATPase. In case of the use of free ATP as a substrate, a

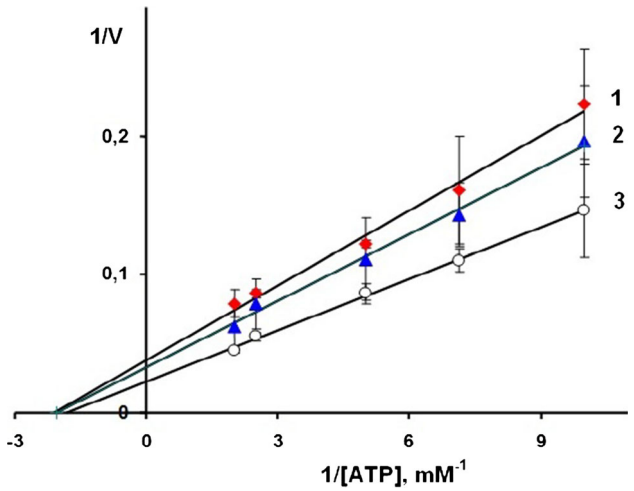

Fig. 7 Dependence of HCO_3^- ATPase activity on ATP concentration in double inverse values at different fixed values of HCO_3 ions: *1* 50 mM; *2* 75 mM; *3* 100 mM

reliable inhibition is achieved, or Mg-independent HCO_3^- ATPase is sensitive to ARL (Tsakadze et al. 2011) (Table 2).

Thus, on the basis of the results obtained, it can be assumed that Mg-independent HCO_3^- ATPase should be attributed to "Ecto" ATPases group.

Discussion

The transport of substances, that is their transmembrane movement, is not only a mandatory link in the metabolism, but also in the vital activity of the living organism as a whole. The active transport of cations and anions is performed by means of molecular mechanisms localized in the membrane ATPases. Nowadays, many ATPases of P-type are found and the establishment of their molecular mechanism and regulation ways take place. On the basis of the researches carried out, some kinetic peculiarities of P-type transport AT Pases were formed (Kometiani and Nozadze 2007):

1 The curve for $V = f(x)$ dependence has a bell-like shape. (x represents transporting ion).
2 Optimal regimen for the system to work provides the inevitable existence of Mg-ATP complex.
3 In the course of catalytic reaction, a phosphorylated intermediate is formed (Gerencser, 1996).
4 It has an oligomeric structure and appears to be a dimer with $\alpha\beta$ subunits.

The experiments carried out on Mg^{2+}-dependent and Mg^{2+}-independent HCO_3^- ATPases have shown that only Mg^{2+}-dependent HCO_3^- ATPases meet the criteria, characteristic of transport ATPases. Below, there is a

Table 1 The dependence of fixed ligand concentrations on regression coefficients

	The concentration of fixed ligand	The coefficients of $1/V = a + bx$ regression		
		a	b	$-a/b$
Fig. 5	[ATP] = 05 mM	0.0180 ± 0.0016	2.860 ± 0.055	0.0063 ± 0.0006
$1/V = f(X)$	[ATP] = 1 mM	0.0216 ± 0.0025	3.707 ± 0.084	0.0058 ± 0.0007
[ATP] = const	[ATP] = 1.5 mM	0.0254 ± 0.0061	4.547 ± 0.006	0.0056 ± 0.0014
Fig. 6	$[HCO_3^-]$ = 50 mM	0.0628 ± 0.0010	0.0126 ± 0.0005	5.413 ± 0.229
1/V = f(ATP)	$[HCO_3^-]$ = 100 mM	0.0407 ± 0.0027	0.0123 ± 0.0012	3.309 ± 0.39
$[HCO_3^-]$ = const				
Fig. 7	$[HCO_3^-]$ = 50 mM	0.0379 ± 0.0057	0.018 ± 0.001	2.106 ± 0.334
1/V = f(1/ATP)	$[HCO_3^-]$ = 75 mM	0.0327 ± 0.0044	0.016 ± 0.001	2.043 ± 0.289
$[HCO_3^-]$ = const	$[HCO_3^-]$ = 100 mM	0.0225 ± 0.0018	0.0124 ± 0.0003	1.815 ± 0.15

Table 2 Effect of ARL67156 on Mg^{2+}-dependent and Mg^{2+}-independent HCO_3^--ion-activated ATPase

	ARL67156, μM				
	0	5	10	20	40
$[Mg^{++}] = 2$ mM**	16.50 ± 1.46	15.25 ± 0.90	14.25 ± 1.25	14.75 ± 1.03	14.50 ± 1.75
$[Mg^{++}] = 0$*	15.0 ± 2.06	9.05 ± 2.02	10.25 ± 2.36	8.50 ± 2.69	7.25 ± 1.25

** $P < 0.05$, * $P > 0.05$

comparative analysis of Mg^{2+}-dependent and Mg^{2+}-independent HCO_3^- ATPases:

1 A bell-like shape which is not characteristic of Mg^{2+}-independent HCO_3^- ATPase and appears to be a necessary condition for all the transport ATPases;
2 Mg-ATP complex is used as a substrate for Mg^{2+}-dependent HCO_3^- ATPases, while Mg^{2+}-independent HCO_3^- ATPase works without Mg^{2+}.
3 A fundamental difference between two enzymes is revealed by means of the analysis of the graphic image of the dependence on the substrate (Figs. 3 and 7). In case of Mg^{2+}-dependent HCO_3^- ATPase, the straight lines are intersecting in the first quarter (Fig. 3), which indicates the orderly connection of the substrate and HCO_3^- to the enzyme molecule: first, the substrate is connected and then HCO_3^-. During the work of Mg^{2+}-independent HCO_3^- ATPase, the lines are intersecting in one point of the abscissa (an error is unreliable) (Fig. 7). The phosphorylated intermediate which is characteristic only of P-type transport ATPases (Gerenser, Gerencser 1996) is seen only in Mg^{2+}-dependent HCO_3^- ATPase (Fig. 2 existence of inflection point).
4 The molecule of Mg^{2+}-dependent HCO_3^- ATPase has an oligomeric structure, which is improved by the existence of turning and inflexion points in this function $V = f(S)$ (Fig. 2). The curve of Mg^{2+}-independent HCO_3^- ATPase has only a turning point (Fig. 6), which excludes the existence of oligomeric enzyme.
5 The use of specific inhibitor: It is known that ARL67156 appears to be a specific inhibitor of "Ecto" ATPases. The experiments carried out on both types of ATPases have shown that ARL67156 inhibits only Mg^{2+}-independent HCO_3^- ATPase, while the effect on Mg^{2+}-dependent HCO_3^- ATPase is unreliable.

Proceeding from the above-said, it may be assumed that Mg^{2+}-independent HCO_3^- ATPase should be attributed to the group of "Ecto" ATPases, while Mg^{2+}-dependent HCO_3^- ATPase appears to be P-type transport ATPase.

As is known from the available literature, ATP, localized in presynaptic vesicles, is released on the membrane surface and fulfills the function of neurotransmitter or neuromediator (Palayoor et al. 1986).

In our opinion, Mg^{2+}-independent HCO_3^- ATPase should control a level of free ATP on the membrane surface.

So, enzymatic system-Mg^{2+}-independent ATPase reaction activated by HCO_3^- anion has been revealed and studied. Mg^{2+}-independent ATPase does not satisfy the conditions necessary for transport ATPases. Presumably, it should be belonged to so-called "Ecto" ATPase group.

Conclusion

In conclusion, the study of Mg-dependent HCO_3^- ATPase reveals an interesting analogy to transport ATPases with general kinetic peculiarities. Mg-HCO_3^- ATPase, like the "p-type" ATPases, is a transport system that effects active movement of HCO_3-anions from the inside to the outside of the synaptosomal and microsomal membranes.

There was not literature evidence about Mg^{2+}-independent ATPase. This enzyme system was discover by us, fundamentally differs from transport ATPases and proceeding from the results obtained should be considered as member of "Ecto" ATPases.

References

Drakulich DA, Spellmon C, Terry DH (2004) Effect of the ecto-ATPase inhibitor, ARL 67156, on the bovine chromatin cell response to ATP. Eur J Pharmacol 485:137–140
Dzneladze S, Tsakadze L, Leladze M, Kometiani Z (2012) Cl anion-dependent Mg-ATPase. J Membr Biol 245(3):151–156
Gassnez D, Komnick H (1981) Inhibition of a Cl^-/HCO_3^- ATPase in the avian salt gland by furosemide and ethacrynic acid Cell. Biol Int Rep 5:239–246
Gerencser GA (1996) The chloride pump: a Cl-translocating P-type ATPase. Crit Rev Biochem Mol Biol 31:303–337
Ivashenko AT (1977) The ATPase system of animal cells stimulated by HCO_3^- ions. Biol Sci 1:25–33
Izutsu KT, Siegel JA (1980) Microsomal HCO_3^-: stimulated ATPase in the rat liver plasma membrane cells. Biochemistry 45:424–429
Kazanova A, Maslova T (1984) The investigation of activation of Na, K-ATPase in the red blood cells of mammals. Biochimiya 49:1089
Kometiani Z (2007) Methods for kinetic analysis of multi-sited enzyme systems. Sakartvelos Matsne, Tbilisi
Kometiani Z, Nozadze E (2007) Kinetic singularities of transport ATPases. Bull Georg Acad Sci 175(4):106–109
Kometiani ZP, Tsakadze LG, Jariashvili TY (1984) Functional significance of the effects of neirotransmitters on the Na, K-ATPase system. J Neurochem 42(5):1246–1250
Leladze M, Tsakadze L, Kometiani Z (2010) The effect of neurotransmitters and synaptic factors on the anion-activated ATPase activity. Georgian Acad Sci Biol B8(3–4):6–10
Levesque SA, Lavoie EG, Lecka J, Bigonnesse F, Sevigny J (2007) Specificity of the ecto-ATPase inhibitor ARL 67156 on human and mouse ectonucleotidases. Br J Pharmacol 152:141–150
Lowry OH, Rosenbrough NY, Randall RY (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–276
Palayoor ST, Seytried DJ, Bernard J (1986) Calcium ATPase activities in synaptic plasma membranes of seizure-prone mice. Neurochemistry 46:1370–1375
Simon B, Thomas L (1972) HCO_3 stimulated ATPase from mammalian pancreas. properties and its arrangement with the enzyme activities. Biochem Biophys Acta 288:434–442
Tsakadze L, Koshoridze N (1976) HCO_3^- stimulated ATPase of rat's brain. Bull Acad Sci Georgian SSR 84(3):701–704
Tsakadze L, Leladze M, Dzneladze S, Kometiani Z (2011) Effect of ARL67156 on Mg-independent HCO_3^--ions activated ATPase. Proc Georgian Natl Acad Sci 37:113–117