APPLICABILITY OF THE INTEGRATED FORM OF THE MICHAELIS – MENTEN EQUATION TO KINETIC STUDIES OF TRANSPORT ATPases

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The present stage of development of membranology is characterized by the fact that important progress in the purification, reconstruction, and investigation of the structure of the transport ATPases has not led to the understanding of the molecular mechanisms of their function [2]. Against this background the return to the classical methods of kinetics, which are being used increasingly often to obtain information on the molecular bases of these systems, becomes understandable. Because of their allosteric nature, transport ATPases do not obey the traditional kinetics but exhibit an abnormal substrate concentration dependence curve with a peak of additional activation in the region of high ATP concentrations. The study of the role of substrate as modifier of ion-transporting systems is of great interest, but the laboriousness of the investigation is a limiting factor.

This paper describes the applicability of a rapid method for determining the kinetic constants of Na,K-ATPase and Ca-ATPase, obtained during the continuous recording of their activity.*

EXPERIMENTAL METHOD

A preparation of the enzyme Na,K-ATPase obtained from bovine brain [8] consisted of a suspension of membrane fragments with Na,K-ATPase activity of 160-180 μmoles inorganic phosphate (P_{in})/mg protein/h at 37°C and was stable during keeping for 4 h at −20°C. ATPase activity was determined from the increase in the P_{in} concentration in the samples after incubation [13] and by a pH-metric method [1] based on the increase in the concentration of H^+ formed during enzymic hydrolysis of ATP at physiological pH values. The increase in the H^+ level was measured continuously by means of the sensitive ÉPP-09 M3 recorder, connected to the pH-340 instrument in a weakly buffered solution (pH 7.0-7.4), in constant-temperature cells with a volume of 6 ml, stirred with a magnetic mixer, at 15, 25, 32, and 37°C. The composition of the incubation mixture (in mM) was: NaCl + KCl 150; $MgCl_2$ 3; ATP-Na_2 0.15-0.6; imidazole 1-3; protein from 20 to 120 μg/ml.

The fraction of sarcoplasmic reticulum (SR) was isolated [4] from the white muscles of a rabbit (hind limbs). The membrane preparation of Ca,ATPase, with an activity of 160-180 μmoles P_{in}/mg protein/h at 37°C, was partially purified by treatment with a solution of the following composition (in mM): EDTA 1; KCl 100; $MgCl_2$ 0.1; histidine 10; pH 8.5, for 1 h at 0-4°C, after which it was sedimented by centrifugation (45,000g, 1 h) and suspended in 10 mM imidazole, pH 7.0. ATPase activity was measured by a pH-metric method in medium (25°C) containing (in mM): KCl 50; $MgCl_2$ 5; EGTA 0.5; $CaCl_2$ 0.4; imidazole 1.5, pH 7.0; protein 250-400 μg/ml. Protein was determined by Lowry's method [9] in the presence of 1% sodium deoxycholate.

The imidazole used was from Merck, the EGTA from Sigma, and the ATP-Na_2 from Reanal, and was recrystallized [5] and converted into the imidazole form on a column with Dowex 50 × 200, 400 mesh. The NaCl and KCl were recrystallized in the presence of EDTA. The ATP concentration in the samples was measured spectrophotometrically.

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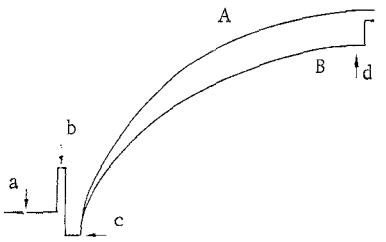

Fig. 1

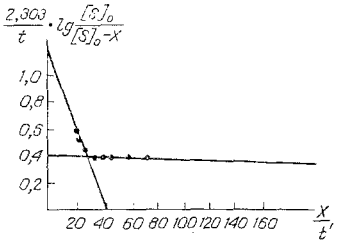

Fig. 2

Fig. 1. Appearance of trace of hydrolysis of ATP by enzyme preparations of Na,K-ATPase (A) and Ca-ATPase (B) with continuous pH-metric recording. a) Zero line; b) titer of phosphate; c) beginning of reaction; d) titer of phosphate repeated at end of reaction.

Fig. 2. Graph of hydrolysis of ATP by Ca-ATPase from SR when integrated form of Michaelis–Menten equation is used.

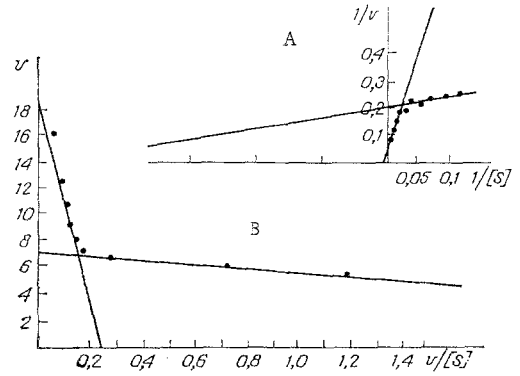

Fig. 3. Graph of hydrolysis of ATP by Ca-ATPase from SR in Lineweaver–Burk (A) and v versus v/[S] (B) coordinates.

TABLE 1. Calculation of Experimental Curve for Hydrolysis of ATP by Ca-ATPase from SR with Continuous Recording of the Process

	Use of integrated form of Michaelis–Menten equation										Use of differential form of Michaelis–Menten equation			
time, min	titer, 0.3 μmole P_{in}/mm width of tape of automatic writer	Quantity of P_{in} formed, μmoles at each minute (P)	Concentration of P_{in} formed, μM [X]	$\frac{X}{t}$	Quantity of ATP hydrolyzed at each minute, μmoles	Concentration of ATP (in μM) remaining at each minute $[S]_0-X_t$	$\frac{[S]_0}{[S]_0-X}$	$\lg\frac{[S]_0}{[S]_0-X}$	$\frac{2{,}303}{t}\cdot\lg\frac{[S]_0}{[S]_0-X}$	P_{in}, μmoles/mg protein/h	$\frac{v}{[S]}$, where $[S]=[S]_0-X$	$\frac{1}{v}$	$\frac{1}{[S]}$
0	29	—	—	—	2,017	336,17	—	—	—	—	—	—	—
1	29	0,641	106,87	106,87	1,014	229,30	1,47	0,1673	0,39	16,02	0,069	0,062	0,004
2	29	1,003	167,17	89,58	0,718	169,00	1,99	0,2989	0,34	12,60	0,074	0,079	0,006
3	29	1,299	216,17	72,05	0,527	120,00	2,80	0,4594	0,35	10,82	0,090	0,092	0,008
4	29	1,490	248,27	62,07	0,362	87,90	3,82	0,5821	0,33	9,30	0,105	0,107	0,112
5	29	1,650	275,77	55,15	0,258	60,40	5,56	0,7451	0,34	8,20	0,135	0,122	0,116
6	29	1,759	287,17	47,86	0,155	49,00	6,86	0,863	0,33	7,30	0,148	0,136	0,020
7	29	1,862	310,27	44,32	0,052	25,90	18,98	1,1106	0,36	6,90	0,266	0,144	0,038
8	29	1,965	327,47	40,33	0,029	8,70	38,64	1,5855	0,45	6,14	0,705	0,162	0,114
9	29	1,986	331,37	36,82	—	4,80	70,03	1,8451	0,47	5,50	1,15	1,181	0,208
10	29	2,017	336,17	33,61	—	—	—	—	—	—	—	0,200	—

EXPERIMENTAL RESULTS

To calculate K_m and V the graph of the increase in reaction product with time during continuous recording of the process was used. Two such traces are shown in Fig. 1 for hydrolysis of ATP in the region of low concentrations (100-600 μM) by preparations of enzymes Na,K-ATPase (A) and Ca-ATPase (B). The angle of slope of the initial part of the acidification curve ought not to be less than 45°. Its value depends on activity of the preparation and the protein content in the sample, the sensitivity of the recorder, and the buffer capacity

TABLE 2. Values of K_m Obtained for Ca-ATPase from SR during Hydrolysis of 150-300 μM ATP and Calculated by Three Different Methods (in μM)

No. Expt.	Use of integrated form of Michaelis–Menten equation		Use of differential form of equation between coordinates			
			Lineweaver-Burk		V from V/[S]	
	K_{m1}	K_{m2}	K_{m1}	K_{m2}	K_{m1}	K_{m2}
1	0,37	11,0	0,65	80,0	0,66	81,0
2	0,37	10,8	1,00	50,0	—	—
3	0,37	10,5	0,68	76,9	—	—
4	0,39	11,9	0,66	77,0	—	—
5	0,38	10,6	0,87	80,0	0,94	79,0
6	—	—	0,87	80,0	0,70	80,8
7	—	—	—	0,92	0,92	69,0
$M \pm m$	0,376±0,007	10,6±0,4	0,79±0,13	74±7,8	0,80±0,12	77,5±4,2

TABLE 3. Values of K_m (in μM) Obtained for Na-K-ATPase during Hydrolysis of ATP at Different Temperatures and with Continuous Recording

37 °C				25 °C			
protein, μg/ml	ATP, μM	K_{m1}	K_{m2}	protein, μg/ml	ATP, μM	K_{m1}	K_{m2}
120	167	18,0	168,0	60	167	5,0	42,0
60	295	20,5	165,0	120	171	4,0	63,0
60	300	24,0	167,0	60	180	3,0	39,0
60	307	21,0	187,0	60	190	4,2	37,0
60	632	21,0	167,0	24	170	3,4	54,0
—	—	20,9±1,3	170,8±6,6	—	—	3,92±0,58	47,0±9,2

of the incubation medium. The sensitivity of the recorder must be such that a change of 0.1 pH unit corresponded to a deflection of the pen of the automatic writer equal to the width of the scale (250 mm). The remaining parameters were chosen so that dissociation of 1-2 micromoles of phosphate caused the pointer of the recorder to be deflected right across the scale. The buffer mixture and protein were introduced into the constant-temperature cell of the pH-meter. After heating of the cell and establishment of a stable zero line, ATP was added. Calibration was carried out at the beginning and end of measurement by the addition of a small portion (0.03 ml) of 10 mM KH_2PO_4. During calculation of ATPase activity a correction was made for the increase in buffer strength of the solution as a result of accumulation of phosphate ions and the change in pH in the course of the reaction. At the beginning of the curve (Fig. 1, A and B), corresponding to saturating concentrations of substrate, the reaction proceeded with constant velocity, after which the velocity decreased as a result of a decrease in substrate concentration, and finally the reaction stopped as a result of exhaustion of substrate. Since in the course of hydrolysis of ATP the quantity of product formed (P) is equimolar to the quantity of substrate hydrolyzed (S), the quantity of phosphate formed (X) is equal to the original quantity of substrate (S_0). Consequently, in the course of hydrolysis of ATP, the reaction velocity at each moment of time (t') will be determined by the quantity of substrate still remaining at that time ($[S]_0 - X$). To calculate V and K_m from the experimental curve, expressing P as a function of t', three methods were used: the differential form of the Michaelis–Menten equation: $V = (V[S])/K_m + [S]$ with different linear transformations in Lineweaver–Burk coordinates and v/v/[S], and also the integrated form of the equation, which when transformed has the appearance: $\frac{2.303}{t} \cdot \log \frac{[S]_0}{[S]_0 - X} = \frac{V}{K_m} - \frac{1}{K_m} \cdot \left(\frac{X}{t}\right)$ [3]. An example of the calculation of the experimental curves is given in Table 1. The concentration of substrate remaining after time t' was calculated from the dependence of the increase in product in the course of the reaction. The graph of dependence of $\frac{2.303}{t} \cdot \log \frac{[S]_0}{[S]_0 - X}$ on X/t was then plotted. In this case a straight line was obtained, with a slope of $1/K_m$, and which intercepted on the abscissa a segment equal to V. In the present experiments, in the case of both Ca-ATPase of SR and of Na,K-ATPase (Fig. 2) we obtained on the graph two populations of points and, correspondingly, two intersecting straight lines and two values of K_m. We next used the differential form of the Michaelis–Menten equation and two different methods of linear transformation.

In both cases, during processing of the experimental curve the value of v was determined at each minute, and [S] corresponded to the concentration of substrate still remaining at each minute $[S] = [S]_0 - X_t$. In the first case, when the graph was plotted between coordinates of 1/v versus 1/[S], two intersecting straight lines were

obtained (Fig. 3). The tangent of the angle of slope of the straight line was K_m/V. In the second case a graph of the dependence of v on v/[S] was plotted (Fig. 3) and two intersecting straight lines also were obtained. The tangent of the angle of slope of these curves was $-K_m$.

All three methods used to represent the velocity of hydrolysis of ATP by transport ATPases graphically had the appearance of two intersecting straight lines (see Figs. 2 and 3), and calculation of K_m gave the values of the two Michaelis constants; values for the first constant, moreover, were practically the same in all methods of calculation (Table 2), whereas values of the second constant agreed in the case of calculation and plotting of the graph between Lineweaver–Burk coordinates and coordinates of v versus v/[S], but they were somewhat smaller when the calculation was done by the integrated form of the Michaelis–Menten equation. Just as in the case of Ca-ATPase from SR, in the case of Na,K-ATPase also values of K_m in full agreement with data in the literature were obtained in the regions of high and low ATP concentrations [5-7, 10, 12, 14, 16]. It was accordingly concluded that this approach is a correct one and that it is preferable because of the ease of obtaining the curve and the simplicity of the calculations, whereby values of K_m and V can be obtained quickly for transport ATPases under different conditions (Table 3).

It is usually considered that the Lineweaver–Burk method requires experimental data of high accuracy, and representation of the dependence of v on v/[S] graphically has the advantage over it because the uniform distribution of the experimental points enables deviations from Michaelis–Menten kinetics to be detected [3]. In the type of calculation shown, it will be clear that the two methods gave equal values of K_m (Table 2). The discovery of two values of K_m in the region of low and high concentrations of ATP for transport ATPases, together with the rapidity of the procedure as used, make this method irreplaceable for the kinetic analysis of allosteric mechanisms of regulation of transport ATPase activity.

During preparation of the manuscript of this article a work was published [15] which described a method of calculating K_m and V for an enzymic reaction catalyzed by AMP aminohydrolase from rabbit muscles, with continuous spectrophotometric recording, and this is further proof of the possibility of the use of rapid recording in order to calculate the kinetic parameters of certain enzymic reactions and of the advantages as regards the speed of determination of K_m and V.

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