

Kinetic Attributes of Na⁺, K⁺ ATPase and Lipid / Phospholipid Profiles of Rat and Human Erythrocyte Membrane

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Kinetic properties of Na⁺, K⁺ ATPase of membranes from rat and human erythrocytes were examined. The enzyme stability decreased with incubation time. The V_{\max} of the human enzyme was about 4 times lower than the values of the rat enzyme. However the energies of activation were higher. Phase transition temperature for the rat and the human enzyme was 24 °C and 17 °C, respectively. The human erythrocyte membranes were characterized by lower total phospholipid and cholesterol contents and were relatively more fluid. The human membranes contained lower proportions of acidic phospholipids which correlated well with the lower V_{\max} of the enzyme; the proportion of lysophosphoglyceride and sphingomyelin was higher in the human membrane.

Introduction

The enzyme Na⁺, K⁺ ATPase plays a crucial role in maintaining intracellular potassium homeostasis (Skou, 1990; Skou and Essman, 1992). High intracellular K⁺ concentration is of importance for a number of intracellular enzymatic reactions including glycolysis and protein synthesis (Skou, 1990).

Kinetic properties of the Na⁺, K⁺ ATPase from kidney, salt gland, brain and heart have been extensively studied (Blazovics *et al.*, 1982; Castillo *et al.*, 1982; Katz *et al.*, 1970; Philipson and Edelman, 1977; Robinson and Flashner, 1979). However not much information along these lines is available for the erythrocyte enzyme (Mishra *et al.*, 1980; Robinson and Flashner, 1979; Rutenbeck, 1979). This may possibly be attributed to the relatively low activity of the pump in the erythrocytes (Deluise and Flier, 1983; Skou, 1990) and the limitations in sensitivity of the methods for measurements of low amounts of phosphate liberated (Fiske and Subbarow, 1925). The number of Na⁺, K⁺ ATPase pumps per RBC is reported to be in the range of 200 to 400 (Deluise and Flier, 1983; Skou, 1990). Because of these inherent limitations various researchers have carried out enzyme assays of the erythrocyte membrane Na⁺, K⁺ ATPase for vary-

ing lengths of time, which can further introduce variability in the results (Baldini *et al.*, 1986, 1989; Levy *et al.*, 1988; Raccach *et al.*, 1992).

We decided to approach this problem by employing a method of phosphate estimation, which is about four times more sensitive (Hurst, 1963) than the conventional method (Fiske and Subbarow, 1925). This allowed us to shorten the assay time to 10–15 min. We studied the kinetic properties of the erythrocyte membrane Na⁺, K⁺ ATPase from rats and humans to find out if any species-specific difference existed. We also examined the lipid / phospholipid profiles of the erythrocyte membranes from the two species and tried to seek correlation between the Na⁺, K⁺ ATPase and the lipid / phospholipid profiles.

Materials and Methods

Chemicals

Sodium salt of vanadium free ATP was purchased from SRL, India. 1,6-Diphenyl-1,3,5 hexatriene (DPH) was purchased from Sigma Chemical Co. U. S. A. All other chemicals were of analytical reagent grade and were purchased locally.

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Preparation of erythrocyte membranes

Citrated blood was washed repeatedly with 0.9% NaCl and the buffy layer overlying the RBC pellet was removed. Washed erythrocytes were lysed by addition of 20 volumes of 5 mM sodium phosphate buffer pH 8.0 and the pellet was collected by centrifuging at 20,000×g for 20 min (Burton *et al.*, 1987). The pellet was successively washed by suspending in 2.5 mM and 1.25 mM sodium phosphate buffer pH 8.0 and by resedimenting as described above. Finally the pellet was washed once with 14 mM tris(hydroxymethyl)aminomethane-HCl buffer pH 7.4 to remove phosphate (Kumthekar and Katyare, 1992). All operations were carried out at 0–4 °C.

Assay of ATPase activity

Measurements of the Mg²⁺ supported Na⁺, K⁺ ATPase activity were carried out at 37 °C. In a total volume of 0.4 ml the reaction mixture contained: 50 mM tris(hydroxymethyl)aminomethane-HCl buffer pH 7.4, 100 mM NaCl, 10 mM KCl and 4 mM MgCl₂ (Kumthekar and Katyare, 1992). 50–100 µg of erythrocyte membrane protein was used as the source of the enzyme.

For these studies we were essentially following the protocol of Skou and co-workers (Skou, 1990; Skou and Essman, 1992), according to which under the conditions one measure the combined effects of Na⁺ and K⁺ on the enzyme activity in the presence of Mg²⁺. The measurements carried out under these conditions are routinely referred to as Na⁺, K⁺ ATPase activity (Skou, 1990; Skou and Essman, 1992).

In preliminary studies, we observed that incubation of 1.0 mM ouabain resulted in about 50% inhibition of enzyme activity in both the membrane systems.

For substrate kinetics studies the concentration of ATP was varied from 0.1 to 2.5 mM.

For determination of optimum activity, ATP concentration was 1.25 mM. (eg. see Fig. 2).

The temperature dependence of the enzyme activity, was measured in the presence of optimum ATP concentration as outlined above over a temperature range of 5 °C to 53 °C with 4 °C steps.

At the end of the incubation period (eg. refer to legends to Figs. 1–3) the reaction was terminated by the addition of 0.1 ml of 5% (w/v) SDS (Kum-

thekar and Katyare, 1992) and liberated phosphorous determined (Hurst, 1963).

Briefly, after terminating the reaction with SDS, the contents were mixed with 1 ml of 3 N sulphuric acid and 0.5 ml of 6.4% ammonium molybdate solution and the volume was brought up to 4.5 ml with distilled water, 0.5 ml of stannous chloride-hydrazine sulphate solution (100 ml of 1 N H₂SO₄ is added to 30 mg of SnCl₂·2H₂O and 30 mg H₂NNH₂H₂SO₄ and the resulting solution is stored in a dark brown bottle at 5 °C) was added and the tubes were vortexed and kept at room temperature for 20 min. The color was read at 660 nm in a colorimeter. The color was found to be stable indefinitely; however the intensity of the blank readings increased after 20 min.

Analysis of substrate kinetics data for determination of K_m and V_{max} (Dixon and Webb, 1979) and of the temperature kinetics data (Raison, 1972) was carried out by employing Sigma Plot, Version 5.0 (Dave *et al.*, 1999).

Lipid analysis

Extraction of total lipids (Bangur *et al.*, 1995; Folch *et al.*, 1957), separation of phospholipid classes by thin layer chromatography (Skipiski *et al.*, 1967) and estimations of cholesterol (CHL) (Zaltkis *et al.*, 1953), and total phospholipid (TPL) phosphorous (Bartlett, 1959) were by the methods cited.

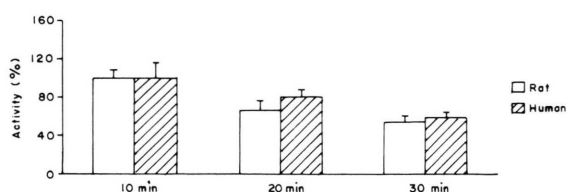


Fig. 1. The effect of duration of incubation time for assay of Na⁺, K⁺ ATPase on the enzyme activity. ATPase activity was determined in the presence of optimum ATP concentration (1.25 mM) as described in the text. The assay time was varied from 10 to 30 min. At the end of incubation period the reaction was terminated with 0.1 ml of 5% (w/v) lauryl sulfate (SDS). Results are mean ± S. E. M. of 8 independent experiments for rat and human RBC membranes respectively. The 10 min incubation values which were taken as 100% were (mean ± S. E. M.) : 2721.9 ± 230.19 and human = 707.6 ± 178.01 nmol Pi liberated / hr × mg protein for rat and human erythrocyte membranes respectively.

Membrane fluidity

The fluidity of the erythrocyte membranes was determined at 25 °C using 1,6 diphenyl 1,3,5 hexatriene (DPH) as the probe, in a RF 5000 Shimadzu Spectrophotofluorimeter following the procedures described earlier in details (Bangur *et al.*, 1995; Mehta *et al.*, 1991; Kaushal *et al.*, 1999).

Protein estimation was according to the method of Lowry *et al.*, (1951) with bovine serum albumin used as the standard.

Results

In the first set of experiments we determined the effect of the duration of the assay period on

the enzyme activity using saturating ATP concentration as in Fig. 2. The enzyme assays were carried out for 10, 20 and 30 min. It can be seen (Fig. 1) that, if the enzyme reactions were carried out for longer durations i.e. 20 or 30 min, the enzyme activity from both the systems decreased to the extent of 20–40% compared to 10 min incubation activity. The data therefore emphasize the point that increased duration of enzyme assay can influence the results adversely.

In the next set of experiments we determined the optimum substrate concentration for the enzyme from human and rat erythrocyte membranes. As can be seen from Fig. 2 the enzyme from the two systems was saturated at the substrate concen-

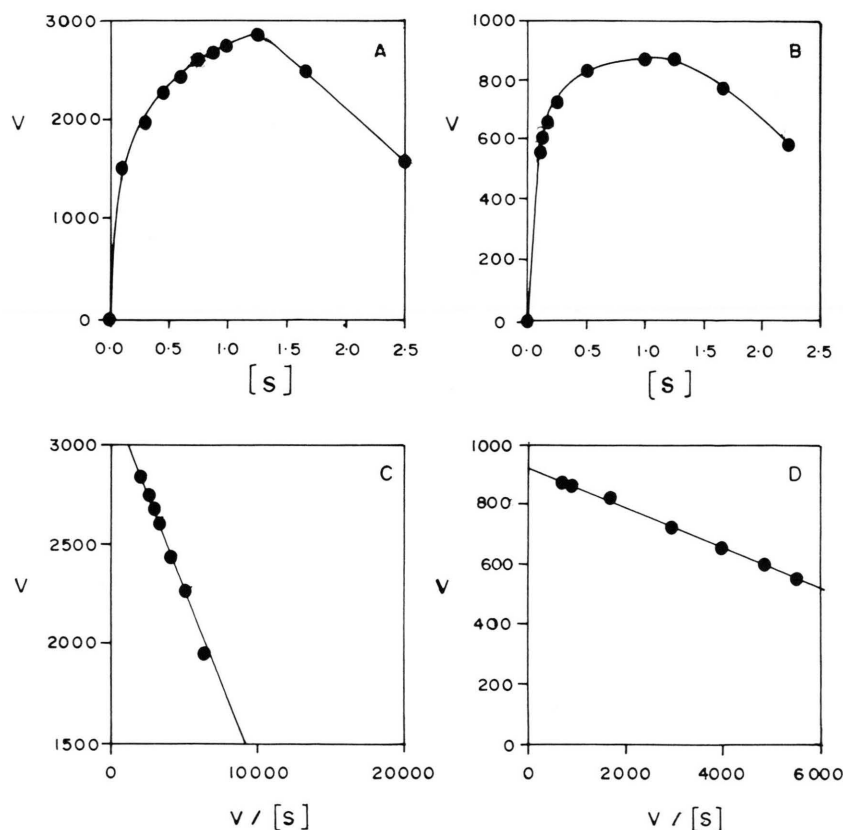


Fig. 2. Typical substrate saturation curves and Eadie-Hofstee plots for rat and human erythrocyte membrane Na⁺, K⁺ ATPase with ATP as the substrate.

The details of the assay system are as given in the text. The reaction was carried out at 37 °C and the incubation time for assay was 10 min. Concentration of ATP ranged from 0.1 to 2.5 mM. The enzyme activity (v) is expressed as $\mu\text{mol Pi liberated} / \text{hr} \times \text{mg protein}$. Substrate saturation curves – A : Rat erythrocytes B: Human erythrocytes. Eadie-Hofstee plots – C: Rat erythrocytes D: Human erythrocytes. The abscissa represents the reaction velocity v, while the ordinate represents the $v/[S]$ ratios. Reaction velocity = $\text{nmol of Pi} / \text{hr} \times \text{mg protein}$. $v/[S]$ = reaction velocity divided by the corresponding substrate concentration.

tration of 1.25 mM. For these experiments (Fig. 2) the incubation time for the enzyme assay was 10 min.

The data on substrate saturation kinetics (Fig. 2) were further analyzed by Eadie-Hofstee plots (Dave *et al.*, 1999; Dixon and Webb, 1979). In both membrane systems there is one kinetic component of Na⁺, K⁺ ATPase. The resulting value for K_m and V_{max} are summarized in Table I. V_{max} and K_m were about 3 to 4 times higher for the rat erythrocyte membrane enzyme thus reflecting the basic differences in the kinetic properties of the enzyme from the two membrane systems. Our value of K_m for human erythrocyte membrane agrees well with that reported by Stojadinovic *et al.* (1996). Kazennov *et al.* (1998) examined species variability of erythrocyte transport ATPase in 5 mammalian species and found that the activity was the highest in mouse and least in the rabbit.

The data on temperature dependence of the enzyme activity are shown in Fig. 3. It is interesting to note that the activity of the enzyme from the rat erythrocyte membranes increased steadily over the temperature range of 5 to 53 °C. In contrast, the enzyme from human erythrocyte membranes showed a temperature optimum at around 40 °C beyond which the enzyme activity decreased. Arrhenius plots for the enzyme from the two membranes are also shown in Fig. 3. Data on the energies of activation and phase transition temperature are given in Table I. In both the cases the plots

were biphasic. However the energies of activation were higher by 37 and 54% for the human enzyme. While the phase transition temperature is lower by 7 °C for the human enzyme. Biphasic response of Na⁺, K⁺ ATPase in human erythrocyte membrane has also been reported by other researchers (Austin *et al.*, 1983).

Since the Na⁺, K⁺ ATPase is dependent on acidic phospholipids: phosphatidylserine (PS) and phosphatidylinositol (PI) for its activity (Robinson and Flashner, 1979) it was of interest to find out if the lipid / phospholipid composition also differs in the two species. From the data in Table I it is evident that the rat membrane contained higher amounts of TPL and CHL (about 80 and 50% higher respectively) resulting in a higher TPL / CHL molar ratio and decreased membrane fluidity in the rat (Table I). The data on phospholipids (Table II) show that the relative proportion of sphingomyelin (SPM) and lysophosphoglyceride (Lyso) were higher in the human membranes (44 to 49% higher), while the proportion of the acidic phospholipids PI, PS and phosphatidic acid (PA) was low (33 to 37% low). Content of phosphatidylcholine (PC) did not differ. Our data on phospholipid composition for rat erythrocyte membranes are in close agreement with those of Kazennov *et al.* (1998).

Table I. Comparison of substrate and temperature kinetics properties of Na⁺, K⁺ ATPase, total phospholipid and cholesterol content and membrane fluidity of rat and human erythrocyte membrane.

	Parameter	Rat	Human
Substrate kinetics properties of Na ⁺ , K ⁺ ATPase	K_m (mM)	0.286±0.023 (7)	0.075±0.010 (5)
	V_{max} (nmol of Pi/ hr × mg protein)	4001.6±299.5 (7)	950.5±134.9 (5)
Temperature kinetics properties of Na ⁺ , K ⁺ ATPase	Energy of Activation (KJ / mol)	E1 26.7±2.05 (6)	36.5±1.15 (7)
		E2 47.5±1.93 (6)	74.3±4.81 (7)
	Phase transition temperature (Tt), °C	24.0±1.15 (6)	16.7±0.93 (7)
Total phospholipid (TPL) µg/mg protein		818.7±14.9 (7)	427.5±30.23 (6)
Cholesterol (CHL) µg/mg protein		360.0±46.50 (7)	220.0±18.95 (6)
TPL / CHL molar ratio		1.24±0.15 (7)	1.01±0.12 (6)
Fluorescence polarization (P)		0.306±0.004 (7)	0.330±0.003 (6)

The experimental details are as described in the text and or in legend to Figs 1 and 3. The results are expressed as mean±S. E. M. Number of independent observations indicated in the parentheses.

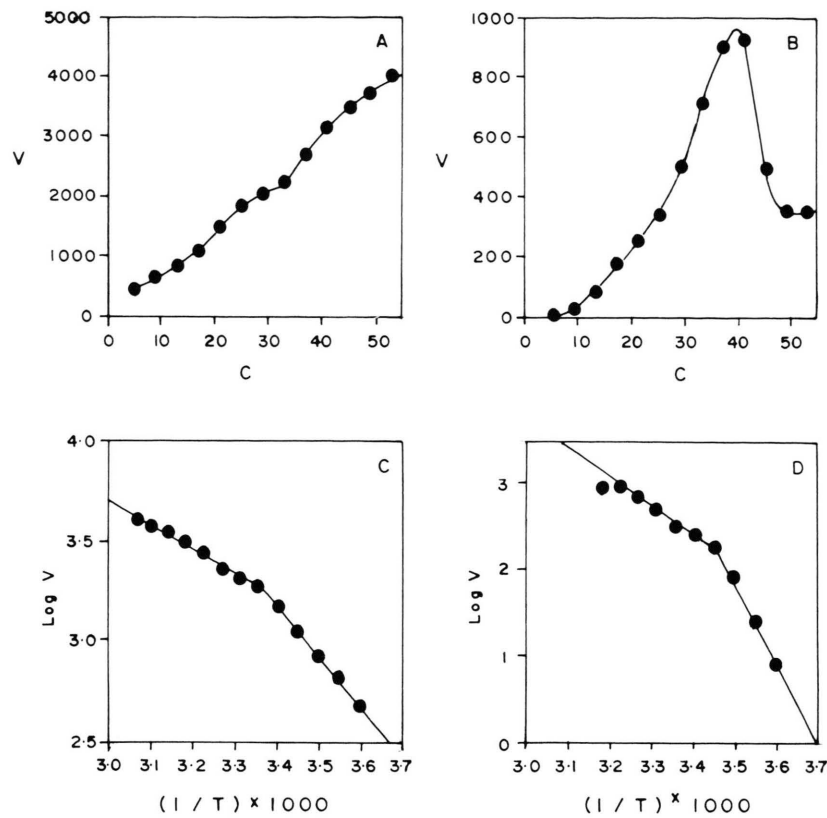


Fig. 3. Typical temperature curve and Arrhenius plots for Na⁺, K⁺ ATPase from rat and human erythrocyte membranes.

Standard assay conditions are as described in the text. The incubation periods were 5° and 9 °C : 30 min; 13°, 17°, 21 °C : 20 min; 25°, 29°, 33 °C : 15 min; 37°, 41°, 45°, 49°, 53 °C : 10 min. Temperature curves – A: Rat erythrocytes B: Human erythrocytes. Arrhenius plots – C: Rat erythrocytes D: Human erythrocytes. The abscissa represents the log of reaction velocity *v*, while the ordinate represents reciprocal of absolute temperature *T* × 1000. Reaction velocity = nmol of Pi / hr × mg protein. Absolute temperature *T* = ° Kelvin.

Table II. Phospholipid composition of rat and human erythrocyte membranes (%).

PL	Rat	Human
Lyso	3.2±0.59	4.6±0.50
SPM	19.5±0.65	28.8±1.02
PC	35.5±0.92	34.7±2.5
PI	9.0±1.45	5.7±1.07
PS	9.6±0.90	6.3±0.79
PE	17.0±0.87	15.5±2.44
PA	6.5±0.66	4.3±0.58

The experimental details are as given in the text. The results are expressed as mean ± S. E. M. of 10 independent observations in each group.

PL, phospholipid; Lyso, lysophosphoglyceride; SPM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA = phosphatidic acid.

Discussion

The present studies were undertaken to delineate the kinetic properties of Na⁺, K⁺ ATPase from erythrocyte membranes. We conducted parallel studies using erythrocyte membranes from rats and humans because the reported half-lives of their RBCs are 60 and 120 days respectively (Alberts *et al.*, 1994), and it seemed likely that differences may also be reflected in the kinetic properties of the membrane bound enzyme, Na⁺, K⁺ ATPase.

Isolated Na⁺, K⁺ ATPase is reported to be relatively stable (Jorgensen, 1982). However our data from Fig. 1 show that the enzyme activity decreases by up to 40% in both the membranes if the enzyme assays were conducted for longer

duration. This observation suggested that dependable results could be obtained only if the assays are performed for short duration. This observation also explains variability in the results of other researchers (Baldini *et al.*, 1986, 1989; Levy *et al.*, 1988; Raccach *et al.*, 1992). The enzymes from the two membrane systems were saturated in the range of 1 mM ATP; higher concentrations were inhibitory (Fig. 2). This agrees well with the reported observations of other researchers (Robinson and Flashner, 1979).

Keeping above observations in mind we restricted our studies to 10 min incubations. The Na⁺, K⁺ ATPase of the two membrane systems comprised of one component in contrast to presence of two components reported for other systems (Robinson and Flashner, 1979). Nevertheless, kinetically the enzyme from the two systems differed. The rat enzyme had K_m and V_{max} values 3 to 4 times higher compared to the human enzyme. The latter observations may possibly relate with the higher metabolic rate and the shorter half-life of the erythrocytes in the rat (Alberts *et al.*, 1994).

The human erythrocyte membrane Na⁺, K⁺ ATPase is believed to be made up of $\alpha 1$ and $\beta 1$ subunits (Jorgenson, 1982). We assume that the same assumption may hold true for the rat erythrocytes. Surprisingly the substrate saturation properties of the two systems differed significantly as discussed above. The differences were further amplified in terms of the temperature kinetics. The human enzyme was inactivated at temperatures beyond 40 °C whereas the rat enzyme was stable up to 53 °C. The energies of activation were higher for the human enzyme. However the phase transition temperature was lower.

It is possible that the observed differences in the K_m value and temperature kinetics could correlate with the lipid / phospholipid makeup of the two

systems. Our studies show that indeed this might be the case. The molar ratios of PC/PE and SPM/PE correlate negatively with the membrane fluidity while the reverse is true for the TPL/CHL molar ratio (Senault *et al.*, 1990; Bangur *et al.*, 1995). The TPL/CHL ratios correlated very well with the fluorescence polarization value (P) given in Table I. Likewise, the PC/PE, SPM/PE ratios (data not given but can be easily calculated from values in Table II) were higher in the rat membranes compared to the human membranes. Taken together with fluorescence polarization, our results suggest that the erythrocyte membranes from the rats were more fluidized. The membrane phase changes have been correlated with the membrane lipid changes (Raison, 1972; Senault *et al.*, 1990). However, validity of such an assumption has been debated (Silvius and McElhaney, 1981). Interestingly we also found that the content of acidic phospholipids i.e. PI and PS of the human membranes was considerably lower, which is consistent with the low enzyme activity in the human membranes (Figs. 1 and 2, Table I). As is well recognized the Na⁺, K⁺ ATPase activity is dependent on the acidic phospholipids (Robinson and Flashner, 1979). Observations correlating the changes in erythrocyte membrane phospholipids and Na⁺, K⁺ ATPase activity have also been reported by other researchers (Arienti *et al.*, 1996; Kakimoto *et al.*, 1995; Panin *et al.*, 1991; Schmalzing and Kutschera, 1982; Vajreswari *et al.*, 1983; Yeagle P. L., 1983).

In conclusion the results of the present studies have clearly demonstrated the substrate and temperature kinetics properties of the rat and human erythrocyte membrane Na⁺, K⁺ ATPase and highlighted the differences in these in the two species in correlation to the membrane lipid / phospholipid composition.

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