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STABILIZATION BY CATIONS OF MICROSOMAL ATPase AGAINST HEAT INACTIVATION

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SUMMARY

(Na^+, K^+, Mg^{2+})-ATPase activity in rat brain microsomes is increasingly inactivated on short incubation at increasing temperatures (25–49 °C) and on prolonged incubation at a single temperature. Inactivation of the Mg^{2+}-ATPase follows that of the (Na^+,K^+)-dependent activity. K^+,Na^+,Mg^{2+} and several other mono- and divalent cations protect microsomal ATPase and *p*-nitrophenylphosphatase from heat inactivation, the divalent cations being more effective. The cation concentration required for protection increases with the temperature applied for inactivation. It is suggested that a membrane conformation accountable for (Na^+,K^+)-ATPase and for *p*-nitrophenylphosphatase activities is stabilized through cation binding.

INTRODUCTION

Metal ions interact with biological membranes and markedly affect their physical and chemical properties. Thus both Ca^{2+} and Mg^{2+} protect red blood cell membranes from damage caused by low ionic strength[1–3] or by sonication[4] while various cations, monovalent as well as divalent, stabilize the structure of chloroplast membranes[5]. We have studied the effect of several cations on rat brain microsomal membranes as revealed by their ATPase, an enzymatic activity highly sensitive to the state of membrane organization[6]. Specifically, the ability of metal ions to protect membranal (Na^+,K^+)-ATPase and *p*-nitrophenylphosphatase against heat denaturation is demonstrated.

MATERIALS AND METHODS

Microsomes

Brains from male albino rats (weight 110–130 g) were homogenized in 0.25 M sucrose containing 1 mM EDTA and 20 mM Tris–HCl (pH 7.4). Material sedimenting at $9000 \times g$ was removed and the supernatant centrifuged at $54000 \times g$ for 90 min. The microsomal fraction thus obtained was treated with NaI, washed in EDTA and suspended in water as described by Nakao *et al.*[7].

Determination of enzyme activities

(Na^+, K^+, Mg^{2+})-ATPase (adenosine 5′-triphosphate phosphohydrolase, EC 3.6.1.3) was assayed in a 1 ml reaction mixture containing 100 mM Tris–HCl, (pH 7.4), 3 mM ATP, 3 mM $MgCl_2$, 100 mM NaCl, 30 mM KCl and 50 μg microsomal protein. The mixture was incubated at 37 °C for 20 min and the reaction stopped by adding cold trichloroacetic acid to a final concentration of 5%. After centrifugation, inorganic phosphorus in the supernatant was determined according to Gomori[8]. In 7 preparations (Na^+,K^+,Mg^{2+})-ATPase activity was 92±4.62 μmoles P_i released/mg protein per h (mean ± S.E.) and Mg^{2+}-ATPase activity, assayed in the absence of NaCl and KCl, was 16±2.67 μmoles P_i released/mg protein per h. *p*-Nitrophenylphosphatase (EC 3.1.3.1) was assayed in a 1-ml reaction mixture containing 10 mM Tris–HCl, (pH 7.4), 3 mM *p*-nitrophenylphosphate (Tris salt), 3 mM $MgCl_2$, 10 mM KCl and 50 μg microsomal protein. The mixture was incubated at 37 °C for 20 min and the reaction stopped by adding cold NaOH to a final concentration of 0.25 M. After centrifugation *p*-nitrophenol in the supernatant was determined by its absorbance at 400 nm. The activity in 6 preparations was 23.54±1.95 μmoles *p*-nitrophenol produced/mg protein per h (mean ±S.E.) and in the absence of KCl 2.34 ± 0.28 μmoles/mg per h.

ATP and *p*-nitrophenylphosphate were purchased from Sigma and used as Tris salts; all chemicals used were analytically pure.

RESULTS AND DISCUSSION

(Na^+,K^+,Mg^{2+})-ATPase and Mg^{2+}-ATPase in microsomes following a short incubation at various temperatures (25–49 °C) is shown in Fig. 1. Suspended in water, ATPase decays exponentially with temperature; the (Na^+,K^+)-dependent

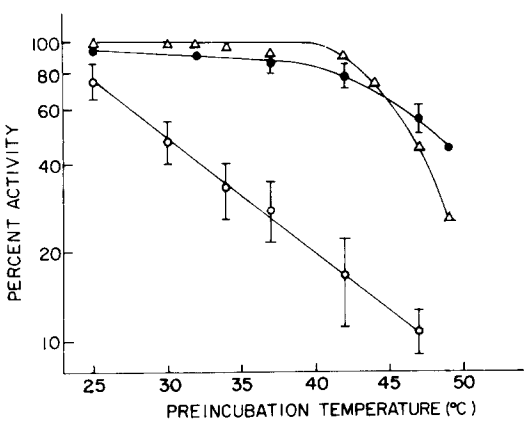

Fig. 1. Effect of temperature and KCl at preincubation on microsomal (Na^+,K^+,Mg^{2+})-ATPase. 50-μg samples of microsomal protein suspended in 0.4 ml water or in 0.4 ml 2.5 mM KCl were incubated for 2 min at the temperatures indicated. Mixtures of buffer, substrate and cations in 0.6 ml were added and enzymic activity determined as described in Methods. (Na^+,K^+, Mg^{2+})-ATPase or Mg^{2+}-ATPase in untreated microsomes was taken as 100% activity. Mean and S.E. are given for experiments carried out 5–7 times. (Na^+,K^+,Mg^{2+})-ATPase: ○—○, preincubation in water; ●—●, preincubation in 0.25 mM KCl. Mg^{2+}-ATPase; △—△, preincubation in water.

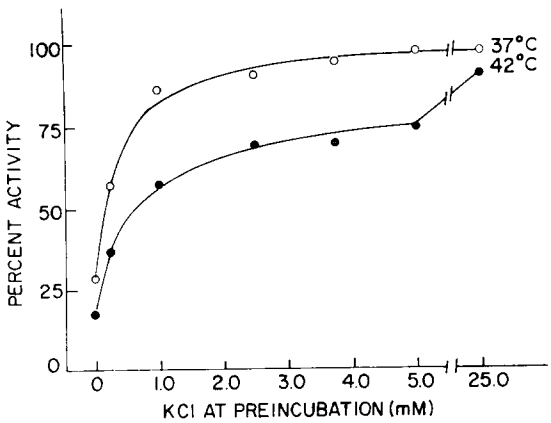

Fig. 2. Effect of KCl concentration on heat inactivation of microsomal (Na^+,K^+,Mg^{2+})-ATPase. Microsomes were preincubated in KCl solutions of varying concentrations for 2 min at the temperature indicated. Details as in legend to Fig. 1, except that the mixtures added for assay contained KCl at the concentration required to make up to 30 mM. ○—○, preincubation at 37 °C; ●—●, preincubation at 42 °C.

activity, about 83% in most preparations, is completely lost at 42 °C while that dependent on Mg^{2+} alone remains unchanged. On further heating Mg^{2+}-ATPase is rapidly inactivated.

A remarkable protection exerted by K^+ on the (Na^+,K^+)-dependent activity at all temperatures tested is demonstrated. As shown in Fig. 2, the effect of K^+ depends on concentration and on the temperature at preincubation; the higher the temperature the more ions are required to obtain a given residual activity. Even at the lowest K^+ concentration tested, 0.25 mM, protection against heat inactivation does occur. The shape of these curves is that of classical binding curves, suggesting that the protective action of the salt is not merely due to increasing ionic strength but is a result of K^+ attachment to the microsomes.

The rate of heat inactivation of two microsomal enzymatic activities, ATPase and *p*-nitrophenylphosphatase, both in water and in 2.5 mM KCl is illustrated by the curves given in Fig. 3. Both activities are markedly stabilized by the salt. In agreement with a report by Tanaka and Mitsumata[9] the kinetics of inactivation of the two activities are similar. This supports suggestions by Robinson[10] and by Garrahan and Rega[11] that K^+-dependent *p*-nitrophenylphosphatase is part of the membranal ATPase system and that both substrates are hydrolyzed at the same enzymic site. The loss of both enzymic activities at 37 °C in the presence of 2.5 mM KCl progresses linearly and is slower than that shown by Atkinson *et al.*[12] for a soluble ATPase preparation preincubated in buffer in the presence of 50 mM NaCl. Heat inactivation in water progresses in a curvilinear fashion, reaching a plateau around 40 min with 40–50% residual activity when preincubated at 30 °C and around 20 min with 5-10% residual activity when preincubated at 37 °C. Since the activity of this particulate microsomal preparation in the absence of Na^+ and K^+ was about 17% of that in their presence, these curves suggest that part of its (Na^+,K^+)-dependent ATPase resists heating at 30 °C and that at higher temperatures its (Na^+, K^+)-dependent ATPase and Mg^{2+}-ATPase are successively inactivated. This latter

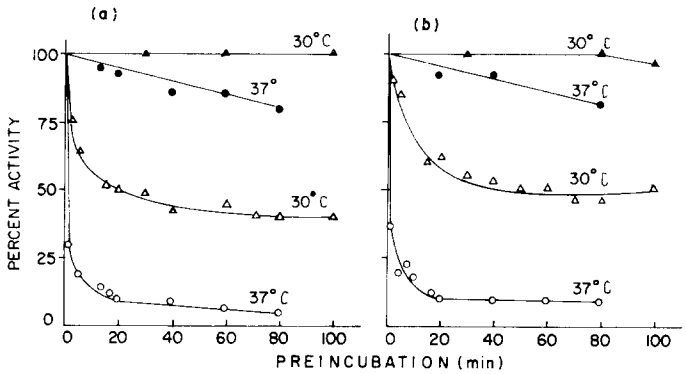

Fig. 3. Effect of KCl on the rate of heat inactivation of microsomal (Na^+,K^+,Mg^{2+})-ATPase and *p*-nitrophenylphosphatase. Microsomes were preincubated in water or in 2.5 mM KCl; incubation period and temperature as indicated. Details as in legend to Fig. 1. (Na^+,K^+,Mg^{2+})-ATPase (a) and *p*-nitrophenylphosphatase (b) were assayed as described in Methods. Preincubation at 30 °C: △—△, in water; ▲—▲, in KCl. Preincubation at 37 °C: ○—○, in water; ●—● in KCl.

suggestion is supported by data given in Fig. 1 on the heat sensitivity in water of both activities. Considering the relative heat stability of the Mg^{2+}-dependent reaction[13–15] and the recent suggestion by Atkinson *et al.*[12] of the subunit structure of transport ATPase and its heat-induced change, this successive inactivation would mean that Mg^{2+}-ATPase is stabilized by an overall conformation of the trimer and that on its dissociation the monomers are exposed to heat inactivation.

Monovalent cations other than K^+ have also been found to stabilize (Na^+, K^+,Mg^{2+})-ATPase, their effect increasing with concentration. These cations are listed in Table I in order of decreasing effectiveness. K^+ and Rb^+ are most efficient

TABLE I

EFFECT OF MONOVALENT CATIONS ON HEAT INACTIVATION OF MICROSOMAL (Na^+, K^+, Mg^{2+})-ATPase

Microsomes were preincubated in a salt solution (type and concn. as indicated) for 2 min at 42 °C. Details as in legend to Fig. 1. ATPase in untreated microsomes, unaffected by the addition of 1 μmole of any of these salts to the assay mixture, was taken as 100% activity. Residual ATPase in microsomes preincubated in water was 15%.

Salt	*Residual ATPase (%)*	
	0.625 mM	*2.5 mM*
KCl	54	72
RbCl	34	66
$TlNO_3$	40	53
NaCl	35	53
CsCl	38	47
LiCl	37	42
NH_4Cl	37	33

TABLE II

EFFECT OF DIVALENT CATIONS ON HEAT INACTIVATION OF MICROSOMAL (Na^+, K^+, Mg^{2+})-ATPase

Microsomes were preincubated in a 0.25-mM solution of the salt indicated for 2 min at 42 °C. Details as in legend to Fig. 1. ATPase in untreated control microsomes, assayed in the presence of 0.1 μmole of each of these salts, was taken as 100% to calculate residual activity. KCl is included for comparison.

Salt	*ATPase* (*μmoles P_i /mg per h*)		*Residual activity* (%)
	Control	*Preincubated*	
None	82	14.5	17.7
$CaCl_2$	67.4	51.2	76
$MgCl_2$	82	57.2	70
$MnCl_2$	65.5	38.5	58.6
$CoCl_2$	74.8	36.2	48.4
$NiSO_4$	73.1	33.7	46
KCl	82	28.6	34.8

while Li^+ and NH_4^+ are least efficient in protecting brain microsomal ATPase from heat inactivation. Except for Na^+, a similar order of effectiveness has been reported by Hegyvary and Post[16] for the interaction of monovalent cations with the ATP binding site on a kidney cortex microsomal preparation.

Robinson[17] has recently suggested that Mg^{2+} and Mn^{2+} specifically bind to rat brain microsomes, thus regulating ATP hydrolysis. Data given in Table II on the effect of these and other divalent cations on heat inactivation of microsomal (Na^+, K^+, Mg^{2+})-ATPase support this suggestion. At 0.25 mM salts of divalent metals protect the enzyme more efficiently than does KCl. Except for Mg^{2+}, their efficiency in protecting microsomes during preincubation at 42 °C (*i.e.* $Ca^{2+} > Mn^{2+} > Co^{2+}$; Ni^{2+}) corresponds to the extent of inhibition induced by them on ATP hydrolysis during the assay and seems to reflect cation affinity to the enzyme.

The experiments described demonstrate that both mono- and divalent metal ions interact with the brain microsomal membrane thus protecting its (Na^+,K^+, Mg^{2+})-ATPase and *p*-nitrophenylphosphatase from inactivation caused by heat. The divalent cations are more effective and the ion concentration required for protection increases with the temperature applied, indicating increasing enzyme stabilization as more ions are bound.

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