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PROPERTIES OF A NON-SPECIFIC NUCLEOTIDASE IN THE MEMBRANE OF RABBIT RED CELLS

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Summary

A non-specific nucleotidase was found in rabbit red blood cell membrane and, using 5'-AMP as substrate, several kinetic parameters for the enzyme were determined. Rabbit red cell ghosts catalyzed the hydrolysis of a wide spectrum of nucleoside 5'-, 3'- and 2'-monophosphates and a limited number of non-nucleotide substrates. All these activities were heat inactivated at the same rate, suggesting that they are the result of catalysis by the same enzyme. The nucleotidase was not dependent on K^+ or Mg^{2+} and was also insensitive to ouabain. Its specific activity and other kinetic parameters were identical in preparation of membranes from both reticulocytes and the mature erythrocytes.

Introduction

The plasma membrane of many cells is known to contain a nucleotidase whose specificity is usually restricted to nucleoside 5'-monophosphates (5'-nucleotidase). Although 5'-nucleotidase activity also occurs in microsomes [1], in the Golgi apparatus [2] and in lysosomes [3], it is often used as a marker of plasma membranes. The physiological role of this enzyme is unclear, but hydrolysis of nucleoside 5'-monophosphates would be a prerequisite for the entry of nucleoside moieties into the cell [4].

The presence of nucleotidases in red cell plasma membrane has been little documented. A 5'-nucleotidase has been detected in avian [5] and human [6] red blood cells. The possible existence of a nucleotidase was investigated in rabbit red cell membrane as a potential marker in the course of maturation of

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these cells. This search led us to the characterization of a non-specific nucleotidase in both young and mature red cells, whose relationship with the above 5′-nucleotidase is discussed.

Materials and Methods

Materials. 2-kg New Zealand rabbits were used. Ribonucleoside 2′-, 3′- and 5′-monophosphates, 2′-deoxyribonucleoside 5′-monophosphates, cyclic adenosine 3′,5′-monophosphate, D-glucose 6-phosphate, D-fructose 1,6-diphosphate, 3-phosphoglycerate, 2,3-diphosphoglycerate, phospho*enol*pyruvate, β-glycerophosphate were purchased from Sigma Chemical Co. EDTA (disodium salt dihydrate) was obtained from Koch-Light and bovine serum albumin from Poviet Producten, N.V. L-(+)-ascorbic acid and most of the organic and mineral chemicals were purchased from Merck. [^{32}P]AMP was obtained from the Radiochemical Center, Amersham.

Preparation of red cell ghosts. Red cell ghosts were prepared essentially according to the procedure of Dodge et al. [7]. Blood was collected into heparin (1000 I.U. for 10 ml blood) by cardiac puncture. All operations were then carried out at 0—4°C. Red blood cells were washed three times with 5 mM phosphate (pH 7.6), 0.15 M NaCl. Each time, the upper part of the pellet was aspirated so that no buffy coat remained. Lysis was initiated by adding 33 vols. of 5 mM phosphate (pH 8), 1 mM EDTA, under mild stirring for 20 min. Red cell ghosts were washed twice with the same volume of the same buffer, then twice with 5 mM phosphate buffer (pH 8) until the pellet (membrane ghosts) was white. Ghosts were stored in aliquots (1 mg protein/ml) at −80°C in 5 mM phosphate buffer (pH 8), 10% glycerol after rapid freezing in liquid N_2. Activity remained unchanged over 8 months. Immediately before use, membranes were washed twice in 5 mM Tris (pH 7.5).

Reticulocyte-rich blood was obtained by injecting rabbits with phenylhydrazine chlorohydrate (10 mg/kg body weight) for 4 days. After a 4-day rest, blood was collected by cardiac puncture. Samples containing less than 60% reticulocytes were not used. Reticulocyte ghosts were prepared in an identical manner. In particular, they underwent the same number of washings. After the last washing, however, they still retained a brownish color (due, probably, to some oxydative degradation products of hemoglobin) that additional washings would have failed to remove.

Assay of nucleotidase. Incubations were carried out for 30 min at 37°C (unless otherwise stated). The reaction mixture (250 μl) contained: 50 mM Tris (pH 7.5), 20 mM KCl, 5 mM magnesium acetate, 100—300 μg protein/ml. The substrate concentration varied from 5 to 80 mM. The reaction was started by addition of the protein (or the substrate in heat inactivation experiments) and stopped by addition of an equal volume of 20% trichloroacetic acid. 400-μl aliquots of the trichloroacetic acid-soluble fraction were used for inorganic phosphate assay. Inorganic phosphate was determined colorimetrically according to the procedure of Chen et al. [8], based on the reduction of a phosphomolybdate complex by ascorbic acid.

Nucleotidase specific activity was expressed as nmol inorganic phosphate liberated per mg protein and per min (nmol · mg^{-1} · min^{-1}).

Results

Kinetic parameters of the nucleotidase using adenosine 5'-monophosphate (5'-AMP) as the substrate

The nucleotidase was initially studied as an adenosine 5'-monophosphatase. The reaction remained linear for at least 30 min and its rate was proportional to the protein concentration (unpublished data). The substrate concentration curve fitted Michaelis-Menten kinetics, with a high apparent Micahelis constant: $K_m = 1.3 \cdot 10^{-2}$ M (This high value made it impossible to reach saturating concentration, e.g. concentrations above $10\,K_m$). Specific activity was 23.5 ± 4 nmol $\cdot$ mg^{-1} $\cdot$ min^{-1} (30 determinations) with the most used concentration (5 mM 5'-AMP) and was approx. 80 nmol $\cdot$ mg^{-1} $\cdot$ min^{-1} at 50 mM 5'-AMP (the highest concentration). Activation energy of the reaction (determined by Arrhenius plots) was 12.4 kcal $\cdot$ mol^{-1} in the 25—41°C temperature range. Heat inactivation started to occur above 51°C and obeyed first-order kinetics; the activation energy of the heat-inactivation reaction was 165 kcal $\cdot$ mol^{-1}.

Special attention was paid to the ionic requirements of the nucleotidase. Using 5'-AMP as substrate, the enzyme was not dependent either on K^+ (0—50 mM) or Mg^{2+} (0—10 mM). Its activity was unaffected by 5 mM Ca^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+}, but was abolished partially (86%) by 5 mM Cu^{2+} and completely by 5 mM Hg^{2+}. 10^{-3} M ouabain did not produce any inhibition in the presence of 20 mM K^+.

Substrate specificity of the nucleotidase

Rabbit red cell ghosts were also able to catalyze the hydrolysis of the

TABLE I

SPECIFIC ACTIVITIES OF THE NUCLEOTIDASE TOWARDS A SERIES OF NUCLEOTIDE SUBSTRATES

Nucleotide substrates (5 mM) were incubated with (a) native membrane ghosts and (b) membrane ghosts which had been preincubated at 54°C for 8 min.

	Specific activity (nmol $\cdot$ mg^{-1} $\cdot$ min^{-1})		
	(a)	(b)	(b)/(a)
5'-AMP	19	6	0.32
5'-GMP	35	13	0.37
5'-CMP	8	2.5	0.31
5'-UMP	8	2.6	0.32
5'-IMP	45	16.3	0.36
3'-AMP	88	33	0.37
3'-GMP	82	30	0.37
3'-UMP	33	10	0.30
2'-AMP	101	n.d.	—
2'-GMP	148	16.5	0.34
2'-UMP	29	9	0.31
cyclic AMP	—		
2'-deoxy, 5'-AMP	46	14	0.30
2'-deoxy, 5'-GMP	116	30	0.26
2'-deoxy, 5'-CMP	49	14	0.33
2'-deoxy, 5'-UMP	21	7	0.33

TABLE II

DETERMINATION OF THE APPARENT K_m FOR VARIOUS NUCLEOTIDE SUBSTRATES

The apparent K_m values were determined using the double reciprocal plots of Lineweaver-Burk.

	K_M (M)
5'-AMP	$1.3 \cdot 10^{-2}$
5'-UMP	$9.5 \cdot 10^{-3}$
3'-AMP	$4.2 \cdot 10^{-3}$
2'-deoxy, 5'-AMP	$1 \cdot 10^{-2}$
2'-deoxy, 5'-UMP	$14 \cdot 10^{-2}$

nucleoside 5'-, 3'- and 2'-monophosphates listed in Table I. Only cyclic adenosine 3',5'-monophosphate was not hydrolyzed. The apparent K_m for some of the substrates are given in Table II. After preliminary heating of the red cell ghosts at 54°C for 8 min, all activities decreased by 67 ± 3% (Table I), suggesting that they result from a single enzyme.

The membranes from red cell ghosts were also able to catalyze the hydrolysis of a few non-nucleotide substrates. However, the specificity of this phosphatase activity was essentially limited. Only β-glycerophosphate (among the non-nucleotide substrates tried) was hydrolyzed at a rate comparable to that of nucleotides (14 nmol · mg^{-1} · min^{-1}). D-Glucose 6-phosphate and phospho*enol*pyruvate were little hydrolyzed. D-Fructose 1,6-diphosphate, 3-phosphoglycerate and 2,3-diphosphoglycerate were not hydrolyzed at all. For β-glycerophosphate, we also determined the apparent K_m: $1.7 \cdot 10^{-2}$ M and the activation energy in the 25—41°C temperature range: 14.1 kcal · mol^{-1}. Hydrolysis

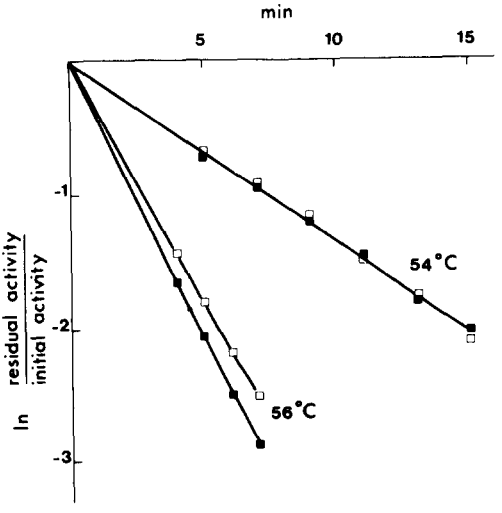

Fig. 1. Heat inactivation kinetics of the nucleotidase activity, as tested with 5'-AMP and β-glycerophosphate. Membrane ghosts were preincubated at 52 or 54°C. At times indicated on the abscissa, aliquots were removed and their activity was determined, with either 50 mM 5'-AMP (□———□), or 50 mM β-glycerophosphate (■———■). The log of the ratio residual activity/initial activity was plotted on the ordinate.

of β-glycerophosphate was not dependent on K^+. It was not inhibited by 10^{-3} M ouabain in the presence of 20 mM K^+. Detailed heat-inactivation experiments with β-glycerophosphate and 5′-AMP showed identical kinetics (Fig. 1), suggesting that phosphatase activity is most likely derived from the same enzyme molecule as nucleotidase activity.

Because of the absence of specificity towards nucleotide substrates and limited specificity towards non-nucleotide substrates, we suggest that the enzyme be termed a non-specific nucleotidase rather than a non-specific phosphatase.

Comparison of the nucleotidase in reticulocyte and erythrocyte membranes

Most of the above parameters and characteristics were also determined in reticulocyte membranes. Using 5′-AMP, the specific activity was 25 nmol · mg^{-1} · min^{-1} (5 mM 5′-AMP), the apparent K_m: $1.4 \cdot 10^{-2}$ M, the activation energy of the heat-inactivation reaction: 118 kcal · mol^{-1}. The ionic requirements and the substrate specificity spectrum were identical.

Discussion

The occurrence of a nucleotidase in rabbit red cell plasma membrane cannot be due to some contamination. Careful removal of the buffy coat prior to hemolysis makes contamination by white cells very unlikely. Electron microscopy showed that ghost preparations from mature erythrocytes were devoid of any organelles; however, preparations from reticulocytes displayed a small amount of damaged mitochondria. Removal of these few mitochondria would have required a purification procedure out of the scope of this work. To our knowledge, nucleoside monophosphatases have not been found in mitochondrial membranes. Furthermore, such membranes would contribute negligible amounts to the protein content of the reticulocyte ghost preparation. We thus considered that minute amounts of mitochondrial debris in reticulocyte ghost preparation would very unlikely be the source of artifacts.

The relationship between this non-specific nucleotidase and the 5′-nucleotidase found in the plasma membrane from most cells is unclear. With regard to 5′-AMP, important differences can be noted. Specific activity was higher by one order of magnitude in the rabbit than in avian erythrocytes [5], but was much lower than in rat liver [9,10]. However, compared to other membrane-bound enzymes of the rabbit erythrocyte : adenylate cyclase [15,16], protein kinases [15], the nucleotidase activity, as expressed per mg membrane proteins, is still rather high. It is interesting to note that the relatively low specific activity of the nucleotidase is not engendered by extensive washing of membrane ghosts. In 'red' ghosts, e.g. ghosts collected immediately after hemolysis (carried out in 5 mM Tris (pH 7.5) for the purpose of this experiment), nucleotidase specific activity was twice as low (35 nmol · mg^{-1} · min^{-1}, using 50 mM 5′-AMP) as in extensively washed ghosts. Therefore, nucleotidase, which underwent a 2-fold purification upon hemoglobin removal, appears to be neither labile, nor loosely bound to the membrane. The apparent Michaelis-Menten constant was much higher than in other cells, where values range between 10^{-6} and 10^{-3} M have been reported [9—13]. The unusually

high K_m cannot be accounted for by some accessibility hindrance of the substrate to the enzyme, because ghosts prepared in the absence of Mg^{2+} are leaky [14]; it questions the relationship between this nucleotidase and the 5′-nucleotidase present in other plasma membranes. One important practical implication of the high apparent K_m is that assays were carried out below saturating concentration (e.g. 10 K_m) of the substrate.

Membrane ghosts were able to hydrolyze a wide series of nucleotide substrates and a limited number of non-nucleotide substrates. One critical question was whether the multiple activities recorded would result from a single enzyme or from a set of nucleotidases and/or phosphatases. Only the availability of pure enzymes could give a definite answer. The purification of membrane-bound enzymes is a difficult task: preliminary attempts at nucleotidase solubilization with Triton X-100 or Lubrol PX led to an important decrease of activity (unpublished data). In the absence of purification, heat-inactivation experiments can provide useful informations. The same heat-inactivation rate observed towards all nucleotide substrates (Table I) suggests that the different activities are due to the same enzyme molecule. Furthermore, it is important to point out that identical heat-inactivation kinetics were obtained with 5′-AMP and β-glycerophosphate (Fig. 1). We therefore thought that it was appropriate to term the enzyme a non-specific nucleotidase, with limited phosphatase activity, rather than a non-specific phosphatase.

Such a broad substrate specificity of the rabbit red cell membrane enzyme contrasts with the restricted specifity of the enzyme in other plasma membranes, usually referred to as 5′-nucleotidase. Specificity was demonstrated to be definitely restricted to nucleoside 5′-monophoshates in several, but not all, cases [9,11,13]. Sometimes, a phosphatase activity was also found to be associated with the nucleotidase [12,17]. However, except for guinea pig polymorphonuclear leukocytes [17], it is not clear whether these two activities result from two separate components. Only in lysosomes has a nucleotidase with a broad specificity been described [3]. At this time, given its high apparent K_m and its broad substrate specificity, the functional relationship between the non-specific nucleotidase of rabbit erythroid cell membranes and the 5′-nucleotidase present in plasma membranes of most non-erythroid cells remains an open question.

Another question which we put forward was whether the non-specific nucleotidase, even though it has limited phosphatase activity, would not result from the $(Na^+ + K^+)$-ATPase. It is accepted that the ATPase reaction first implies the Na^+-activated formation of a phosphorylated intermediate of the enzyme; the second part of the reaction then includes a K^+-activated dephosphorylation [18], which is ouabain sensitive. The absence of responsiveness of rabbit red cell membrane nucleotidase (with either 5′-AMP or β-glycerophosphate) to Mg^{2+}, K^+ and ouabain rules out the hypothesis that the nucleotidase is the expression of the $(Na^+ + K^+)$-ATPase.

It is remarkable that nearly all parameters of the nucleotidase, especially the specific activity, were the same in the erythrocyte and the reticulocyte. The activities of most cytoplasmic enzymes of the red cell are known to decline upon red cell ageing. The same phenomenon seems to hold for several membrane enzymes and receptors [15,16,19,20]. We do not know whether

such an exception has a physiological meaning in so far as the function itself of the nucleotidase is not understood. Another intriguing point is that no such nucleotidase was found in red cells from other mammalian species: sheep, dog, rabbit and rat (unpublished data).

Further work will be necessary to elucidate the physiological role of this non-specific nucleotidase, to localize its active center in one of the membrane surfaces and finally to achieve its purification.

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