5'-Nucleotidase activities in human erythrocytes

Identification of a purine 5'-nucleotidase stimulated by ATP and glycerate 2,3-bisphosphate

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A purine 5'-nucleotidase has been separated by DEAE-Trisacryl chromatography from other 5'-nucleotidase activities present in human haemolysates and purified approx. 30000-fold by subsequent chromatography on Blue Sepharose. The enzyme has an M_r of around 250000, displays hyperbolic substrate-saturation kinetics and hydrolyses preferentially IMP, GMP and their deoxy counterparts. It is much less active with AMP and dAMP. The purine 5'-nucleotidase is inhibited by P_i , and is strongly stimulated by ATP, dATP and GTP, and by glycerate 2,3-bisphosphate. Stimulators decrease K_m and increase V_{max} . Glycerate 2,3-bisphosphate is the most potent stimulator of the enzyme and, under physiological conditions, over-rides the influence of the other effectors. Glycerate 2,3-bisphosphate also influences the binding of the enzyme to DEAE-Trisacryl, as evidenced by the different elution profile obtained with fresh as compared with outdated blood. It is concluded that the glycerate 2,3-bisphosphate-stimulated purine 5'-nucleotidase is responsible for the dephosphorylation of IMP and GMP, but not of AMP, in human erythrocytes.

INTRODUCTION

Dephosphorylation constitutes an obligatory step in the catabolism of various species of nucleoside 5'-monophosphates present inside the cells. In the older literature, the membranous ecto-5'-nucleotidase was generally considered responsible for the catabolism of these compounds. However, more recent studies show that, quite logically, this role is most probably assumed by cytosolic enzymes. First described by Itoh *et al.* [1] and by Fritzson [2], these soluble 5'-nucleotidases have been shown to possess complex regulatory properties, including stimulation by nucleoside triphosphates and inhibition by P_i [3–5]. Whether distinct cytoplasmic 5'-nucleotidases exist for the specific or preferential degradation of the various 5'-nucleotide species remains, however, less clear.

In mature erythrocytes, even more than in other cell types, adenine nucleotides constitute the most abundant nucleotide compounds. 5'-Nucleotidase(s) intervene(s) in their catabolism by dephosphorylating either AMP or its deamination product IMP, depending on the experimental conditions [6–9]. Yet the precise identity of the enzyme(s) responsible for this process has not been determined with certainty. A pyrimidine 5'-nucleotidase, characterized by Paglia & Valentine [10], was found to be inactive on purine 5'-ribonucleotides. Moreover, haemolysates from subjects with a hereditary haemolytic anaemia caused by a deficiency of pyrimidine 5'-nucleotidase remain able to dephosphorylate several pyrimidine 5'-deoxyribonucleotides [11,12] and purine 5'-ribo- and deoxyribo-nucleotides [13] at normal rates. The substantially higher activities recorded in the last study [13] with deoxyribonucleotides as compared with ribonucleotides has led to the suggestion that the erythrocyte 5'-nucleotides, except for the pyrimidine 5'-ribonucleotides, are degraded by a deoxyribonucleotidase.

The present study reports the partial purification and characterization of a third 5'-nucleotidase in human erythrocytes, which hydrolyses preferentially the purine 5'-ribonucleotides IMP and GMP and their deoxy-counterparts. Its kinetic characteristics, when compared with those of other 5'-nucleotidase activities present in crude haemolysates, suggest that it is responsible for the dephosphorylation of IMP and GMP, but not of AMP, in human erythrocytes. Part of this work has been presented at a symposium [14,15].

MATERIALS AND METHODS

Chemicals

Nucleotides, nucleosides and bases, and glycerate 2,3-bisphosphate (2,3-DPG) were from Boehringer (Mannheim, Germany). Deoxynucleotides and bovine serum albumin (fraction V) were from Sigma (St Louis, MO, U.S.A.). [$8\text{-}^{14}\text{C}$]AMP (59 Ci/mol), [$8\text{-}^{14}\text{C}$]IMP (53 Ci/mol), [$U\text{-}^{14}\text{C}$]GMP (551 Ci/mol), [$U\text{-}^{14}\text{C}$]CMP (526 Ci/mol), [$5\text{-}^3\text{H}$]dUMP (10.6 Ci/mmol) and [$8\text{-}^{14}\text{C}$]adenosine (54 Ci/mol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Coformycin {(*R*)-3-(β -D-erythropentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol} was a gift from Professor H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan). DEAE-Trisacryl M was from LKB (Bromma, Sweden). Blue Sepharose, Superose 6HR 10/30, Sephacryl S-300, Sephadex G-25 (medium grade) and the gel-filtration calibration standards were from Pharmacia (Uppsala, Sweden). Dye reagent concentrate and bovine γ -globulin were from Bio-Rad (Richmond, CA, U.S.A.). Dithiothreitol was purchased from Janssen Chimica (Beerse, Belgium). All other chemicals were analytical grade.

Abbreviation used: 2,3-DPG, glycerate 2,3-bisphosphate.

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Enzyme assays

5'-Nucleotidase activities were measured by the release of either the nucleoside or P_i , the latter method being used at substrate concentrations above 2 mM or when no radioactive substrate was available. Incubations were performed at 37 °C in a medium containing 10 mM-MgCl₂, 50 mM-Tris/HCl buffer, pH 7.2, or 50 mM-Mes, pH 6.3, various concentrations of substrate and other additions as indicated. To measure the release of nucleosides, assays were performed with radioactive 5'-nucleotides, in a total volume of 50 or 100 μ l. The reactions were terminated by spotting 10 μ l of the incubation medium on cellulose t.l.c. plates, on which carrier solutions (50 nmol) of the 5'-nucleotide substrate and of its corresponding nucleoside and base had been applied. After development in butan-1-ol/methanol/water/conc. NH₃ (60:20:20:1, by vol.) [16], the enzymic activity was calculated from the quantity of radioactive nucleoside formed. When crude preparations were assayed, the radioactivity in the base was also taken into account. When the dephosphorylation of AMP was measured in the latter preparations, 100 μ M-coformycin was added to the assay mixture in order to inhibit both adenosine deaminase and AMP deaminase [17,18]. To measure the release of P_i , assays were performed in a total volume of 0.5 ml and terminated by addition of 0.25 ml of ice-cold 0.6 M-trichloroacetic acid. Reagent blanks were incubated in parallel, containing all ingredients of the reaction mixture, except for the absence of substrate, which was added after deproteinization. The amount of P_i liberated was determined as described by Itaya & Ui [19].

The activity of acid phosphatase was determined by measuring the release of P_i from 10 mM- α -glycerophosphate in 50 mM-Mes buffer, pH 5. The activity of adenosine kinase was determined by measuring the incorporation of 50 μ M-[8-¹⁴C]adenosine into AMP, in the presence of 3 mM-MgATP and 50 mM-Tris/HCl, pH 7.2. Adenylate kinase was assayed by the method of Adam [20]. The activities of AMP deaminase, adenosine deaminase and nucleoside phosphorylase were measured by radiochemical methods described by Vincent *et al.* [21].

Preparation of haemolysates

Fresh blood taken from a cubital vein of healthy human volunteers was collected either on heparin or in ACD (acid-citrate-dextrose). Outdated blood had been stored in ACD. After centrifugation and removal of plasma and buffy coat, the erythrocytes were resuspended in 0.9% NaCl and freed of leucocytes and platelets as described by Beutler *et al.* [22]. After microscopic verification that over 95% of leucocytes and thrombocytes had been removed, the erythrocytes were washed three times in 0.9% NaCl, and thereafter haemolysed by addition of 4 vol. of 10 mM-Tris/HCl buffer, pH 7.2, containing 1 mM-EDTA and 1 mM-dithiothreitol (buffer A). Where indicated, the haemolysates were dialysed overnight against 500 vol. of buffer A; this first dialysis was followed by a second one, of 2 h duration.

Purification procedure

DEAE-Trisacryl chromatography. A 1:5 haemolysate, prepared from 1 unit (350–500 ml) of blood collected in ACD, was mixed for 1 h with an equal volume of DEAE-Trisacryl, equilibrated and diluted 1:5 with buffer A.

After abundant washing with buffer A to remove haemoglobin, the resin was poured into a 1.6 cm or 2.6 cm $\times$ 40 cm chromatography column. The column was eluted with a 0–400 mM linear gradient of NaCl in buffer A, at a flow rate of 60–80 ml/h.

Chromatography on Blue Sepharose. Fractions containing the purine 5'-nucleotidase were pooled, and 5 ml of this pool were applied to a 0.4 cm $\times$ 6 cm column, containing 0.2 ml of the resin. After rinsing with 5 $\times$ 1 ml of buffer A, containing 0.5 M-NaCl, the purine 5'-nucleotidase was eluted with 2 M-NaCl in buffer A. The eluate, collected in albumin (1 mg/ml) to stabilize the enzyme, was dialysed against 500 vol. of buffer A for 1 h to remove NaCl, which inhibits its activity.

Determinations of M_r

These were performed on DEAE-Trisacryl eluates after pooling and 5–10-fold concentration of active fractions on Amicon YM 10 filters. Gel filtration was performed as indicated in the Results section either on a Superose 6HR 10/30 column (1 cm $\times$ 30 cm) or on a Sephacryl S-300 column (1.6 cm $\times$ 40 cm). Elution was accomplished with buffer A containing 200 mM-NaCl, with or without 2,3-DPG as indicated. Fractions of low protein content were collected in albumin (1 mg/ml). Apparent M_r values were determined by comparison of the elution profile with those of the following calibration standards: ferritin (M_r 440 000), aldolase (160 000), ovalbumin (45 000) and cytochrome *c* (12 500).

Analytical methods

2,3-DPG was measured as described by Michal [23]. Protein was determined by the method of Bradford [24], with bovine γ -globulin as the standard.

RESULTS

5'-Nucleotidase activities in dialysed haemolysates

Widely different rates of dephosphorylation were recorded when various 5'-nucleotides, at a concentration near the physiological range, namely 0.25 mM, were incubated at the physiological pH, 7.2, with dialysed haemolysates (Table 1). The activities recorded with the pyrimidine 5'-ribonucleotide CMP, and with the deoxy-ribonucleotides dUMP and dIMP, were 8–50-fold higher than those measured with purine ribonucleotides.

The observation, described below, that 2,3-DPG, the main phosphate ester present in human erythrocytes, stimulates the partially purified purine 5'-nucleotidase, prompted a study of its effect on the various 5'-nucleotide-dephosphorylating activities present in the dialysed haemolysate. As also shown in Table 1, the dephosphorylation of the pyrimidine nucleotides CMP and dUMP was not influenced by 5 mM-2,3-DPG, and that of dIMP was increased, although not significantly. In contrast, the dephosphorylation of IMP and GMP was stimulated respectively 2- and 5-fold, and that of AMP was significantly enhanced by 20%.

Separation of 5'-nucleotidase activities by DEAE-Trisacryl chromatography

Fig. 1 shows elution profiles obtained when a haemolysate, prepared from fresh blood and freed from haemoglobin as described in the Materials and methods

Table 1. 5'-Nucleotidase activities in dialysed human haemolysates

Activities were determined at 0.25 mM substrate concentration and pH 7.2. With dIMP, the release of P_i was measured; with the other 5'-nucleotides, the radiochemical method was used. Values shown are means $\pm$ S.E.M. for $n = 4$. Differences between experimental conditions were tested for significance by the paired t test: n.s., not significant.

Substrate (0.25 mM)	5'-Nucleotidase activity (nmol/min per g of haemoglobin)		Stimulation (fold)	<i>P</i>
	No addition	+ 2,3-DPG (5 mM)		
CMP	96 $\pm$ 18	97 $\pm$ 20	—	n.s.
dUMP	87 $\pm$ 8	89 $\pm$ 11	—	n.s.
dIMP	137 $\pm$ 11	182 $\pm$ 14	1.3	< 0.1
IMP	11 $\pm$ 2.3	22 $\pm$ 1.8	2.0	< 0.05
GMP	2.7 $\pm$ 0.7	13 $\pm$ 0.5	4.8	< 0.001
AMP	5.0 $\pm$ 0.8	6.0 $\pm$ 0.8	1.2	< 0.05

Fig. 1. DEAE-Trisacryl chromatography of 5'-nucleotidase activities in human erythrocytes

The column, prepared as described in the Materials and methods section, was eluted with the linear gradient of NaCl, shown in the centre panel, and 6 ml fractions were collected. The activities toward dUMP, CMP, IMP and AMP were measured at 0.25 mM substrate concentration and pH 7.2 by the radiochemical method. The activity toward 0.25 mM-dIMP was measured by the release of P_i . Acid phosphatase was assayed at pH 5 with 10 mM- α -glycerophosphate (α -GP).

section, was applied to a DEAE-Trisacryl column and washed off with a linear gradient of NaCl. In this system, 5'-nucleotidase activities, measured with the same substrates and at the same pH as in Table 1, were released from the column in two peaks. Peak I, eluted at 50 mM-NaCl, also contained most of the proteins and displayed maximal activity with dIMP, dUMP and CMP. Much

lower activities were measured with IMP and AMP. A slight retardation of the activity toward dIMP, IMP and dUMP, compared with that toward CMP and AMP, was reproducibly recorded in peak I. The activity of the acid phosphatase present in human erythrocytes [25], measured with α -glycerol phosphate as substrate, was also eluted in peak I. Peak II was eluted at 240 mM-NaCl,

and contained about 10 % of the proteins applied to the column. It displayed the highest activity with IMP and dIMP, and about 20-fold lower activities with dUMP, CMP and AMP. Peak II also contained 2,3-DPG, which was eluted from the column at NaCl concentrations above 150 mM.

5'-Nucleotidase activities of peak I

Further analysis of the 5'-nucleotidase activities present in peak I was performed both at pH 7.2, the physiological pH of erythrocytes, and at pH 6.3, the optimum pH of cytosolic 5'-nucleotidase in various tissues [1-3] and of the deoxyribonucleotide-dephosphorylating activity of human erythrocytes [12]. Substrate concentration was 2.5 mM. As shown in Table 2, the highest rates of hydrolysis were measured with deoxypurine and deoxypyrimidine nucleotides. All activities, except those with CMP, were higher at pH 6.3 than at pH 7.2. Detailed pH/activity curves (not shown) showed that maximal activity was observed at pH 7.5 with CMP, at pH 5.75 with IMP, dIMP and dUMP and at pH 6 with AMP. The rate of dephosphorylation of these substrates was not influenced by the addition of 5 mM-2,3-DPG (results not shown). Molecular sieving of peak I through a Superose 6HR column showed that the dephosphorylating activities toward CMP and AMP nearly co-eluted, with an apparent M_r of 35000; the dIMP-, IMP- and dUMP-dephosphorylating activities were slightly heavier, with an apparent M_r of 45000; the acid phosphatase activity was eluted with an apparent M_r of 70000 (results not shown).

5'-Nucleotidase activities of peak II

Purification and molecular properties. When peak II material was applied on a Blue Sepharose column, followed by washing with 0.5 M-NaCl, 5'-nucleotidase was adsorbed on the resin, whereas more than 98 % of the proteins were not (Fig. 2). Elution with 2 M-NaCl released the 5'-nucleotidase activity. The low (5-20 %) recovery of the enzymic activity when measured at this step was increased to 50-60 % by inclusion of 3 mM-2,3-DPG in the assay. This suggested that the 2,3-DPG

Fig. 2. Elution profile of the purine 5'-nucleotidase from the Blue Sepharose column

Pooled fractions (total volume 5 ml) of peak II from the DEAE-Trisacryl column, were applied and eluted as described in detail in the Materials and methods section. Fraction volume was 1 ml. The activity was measured by the radiochemical method at pH 7.2 with 0.25 mM-IMP as substrate in the absence (○) and in the presence (●) of 3 mM-2,3-DPG.

Table 2. Substrate specificity and influence of pH on the 5'-nucleotidase activities eluted in peak I of the DEAE-Trisacryl column

Experiments were performed on pooled fractions after filtration on Sephadex G-25. Activities were measured at 2.5 mM substrate concentration by the release of P_i in 50 mM-Mes buffer, pH 6.3, or in 50 mM-Tris/HCl buffer, pH 7.2. Values shown are means of two determinations.

Substrate	Activities (nmol/min per mg of protein)	
	pH 6.3	pH 7.2
CMP	8	10
dUMP	46	23
dIMP	55	24
IMP	6.5	2.0
GMP	2.7	1.5
AMP	7.7	1.7

present in peak II of the DEAE-Trisacryl eluate of the haemolysate prepared from fresh blood was separated from the enzyme by the Blue Sepharose chromatography. Measurements of the concentration of 2,3-DPG in the column eluates confirmed this hypothesis (Fig. 2).

Table 3 summarizes the isolation procedure, which yielded an approx. 30000-fold-purified enzymic activity, as measured with IMP as substrate. The enzyme was free of measurable acid phosphatase, AMP deaminase, adenosine deaminase, nucleoside phosphorylase, adenylate kinase and adenosine kinase activities. Its apparent M_r , estimated by gel filtration on Sephacryl S-300, was 250000. Owing to the low initial activity and limited stability of the enzyme, attempts at further purification have been unsuccessful.

Kinetic properties. (1) Effect of Mg^{2+} ions and of pH. The 5'-nucleotidase purified from peak II was completely Mg^{2+} -dependent. An apparent K_s of 1.7 mM- $MgCl_2$ was measured at 1 mM-IMP (result not shown). The enzyme displayed a fairly sharp activity optimum at pH 6.3, and its activity decreased to 50 % thereof at pH 7.2 (results not shown).

(2) Influence of substrate. As depicted in Fig. 3, substrate saturation curves for IMP and GMP were hyperbolic in the absence of effectors; apparent K_m values were 0.4 and 0.8 mM respectively. Because of the low activity of the enzyme with AMP, the apparent K_m for this substrate could not be precisely determined. When measured at 0.25 mM concentration, the activity of

Table 3. Purification of the purine 5'-nucleotidase from human erythrocytes

Activities were measured at 0.25 mM-IMP and pH 7.2, in the presence of 3 mM-2,3-DPG, by the radiochemical method. Values shown are calculated by making the assumption that all the enzyme obtained at the DEAE-Trisacryl step is used for the Blue Sepharose step.

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)	Purification (fold)
Haemolysate	109650	1572	0.014	100	1
DEAE-Trisacryl (peak II)	81	350	4.3	22	308
Blue Sepharose	0.41	183	446	11.6	31880

Fig. 3. Influence of substrate concentration on the activity of the purine 5'-nucleotidase purified from human erythrocytes

Activities were measured at pH 6.3 by the radiochemical method in the absence (○, □, △) and in the presence (●, ■, ▲) of 3 mM-2,3-DPG.

the enzyme on XMP, dIMP and dGMP was about half of that recorded with IMP and GMP, and was barely detectable with dAMP and with pyrimidine ribo- and deoxyribo-nucleotides (results not shown). However, at 2.5 mM substrate concentration, activities toward the last nucleotides, although still lower than those recorded with IMP, GMP and their deoxy counterparts, became measurable (Table 4, left column).

(3) Effectors. As shown in Table 5, similarly to other cytosolic 5'-nucleotidases, the activity of the purified erythrocytic 5'-nucleotidase, measured at sub-saturating concentrations of IMP and AMP, was greatly stimulated by all the nucleoside triphosphates tested, the most potent ones being ATP and dATP. ADP was also stimulatory, although to a smaller extent. Also like other cytosolic 5'-nucleotidases, the enzyme was inhibited by

P_i . As mentioned above, and in contrast with the 5'-nucleotidase activities of peak I, the activity of the enzyme purified from peak II was stimulated by 2,3-DPG, to a similar extent as by ATP. As shown in Fig. 4, with 0.2 mM-IMP as substrate, half-maximal stimulation was obtained at 0.1 mM-2,3-DPG and at 0.5 mM-ATP, both saturation curves being slightly sigmoidal. At sub-saturating, but not at saturating, concentrations, the two stimulators had additive effects (not shown). 2,3-DPG also stimulated the dephosphorylation of the other substrates of the enzyme: that of XMP, dIMP and dGMP was stimulated approx. 10-fold by 3 mM-2,3-DPG at 0.25 mM concentration (results not shown) and 2–5-fold at 2.5 mM concentration (Table 4, right column). The dephosphorylation of CMP, UMP, dCMP, dUMP, dTMP and dAMP at 0.25 mM concentration remained

Table 4. Substrate specificity of the purine 5'-nucleotidase purified from human erythrocytes

Activities were measured at 2.5 mM substrate concentration by the release of P_i in 50 mM-Mes buffer, pH 6.3. Values shown are means of two determinations.

Substrate	Activities (nmol/min per mg of protein)	
	No addition	+2,3-DPG (3 mM)
CMP	28	278
UMP	58	405
dCMP	12	78
dUMP	32	258
dTMP	23	143
dIMP	132	493
dGMP	98	480
dAMP	22	155
IMP	152	468
GMP	132	557
XMP	173	398
AMP	28	280

Table 5. Influence of various compounds on the activity of the purine 5'-nucleotidase purified from human erythrocytes

Activities were measured at 0.2 mM substrate concentration and pH 6.3 with the radiochemical method. Additions were at 1 mM concentration. Values shown are means of two determinations.

Additions	Activities (nmol/min per mg of protein)	
	IMP	AMP
None	47	2.0
ATP	470	17
dATP	495	28
GTP	243	3.3
ITP	190	2.5
CTP	122	2.7
UTP	82	1.7
ADP	143	3.2
GDP	47	0.7
P_i	7	0.2
2,3-DPG	497	36
2-Phosphoglycerate	93	2.5
3-Phosphoglycerate	63	2.0

barely detectable in the presence of 3 mM-2,3-DPG (results not shown), but was stimulated 6–10-fold at 2.5 mM concentration (Table 4, right column).

More detailed kinetics (Fig. 3) demonstrated that the stimulatory effect of 2,3-DPG was of mixed type: with IMP as substrate, the apparent K_m decreased from approx. 0.4 mM to about 0.04 mM, and V_{max} increased 3–4-fold; with AMP at 2 mM concentration, the enzyme activity increased 25-fold in the presence of 3 mM-2,3-

Fig. 4. Effect of 2,3-DPG and of ATP on the activity of purified purine 5'-nucleotidase

Activities were measured at pH 6.3 with 0.2 mM-IMP as substrate, by the radiochemical method, in the presence of 2,3-DPG and ATP as indicated.

Fig. 5. Effect of P_i on the activity of the purified purine 5'-nucleotidase

Activities were measured by the radiochemical method at pH 6.3 with 0.2 mM-IMP as substrate, without and with the addition as indicated of 1 mM-ATP, 3 mM-2,3-DPG or both.

Fig. 6. DEAE-Trisacryl chromatography of the 5'-nucleotidase activities toward IMP in outdated (a) as compared with fresh (b) erythrocytes

Columns, prepared as described in the Materials and methods section, were eluted with linear gradients of NaCl. Fraction volume was 3 ml in (a) and 6 ml in (b). Activities were measured at 0.2 mM-IMP and pH 7.2, in the absence (○) and in the presence (●) of 3 mM-2,3-DPG, by the radiochemical method.

DPG, reaching about one-third of the velocity measured with IMP and GMP under the same conditions. Similar mixed stimulatory effects were obtained with ATP (results not shown). At 0.2 mM-IMP, half-maximal inhibition was obtained at 0.25 mM- P_i in the absence of other effectors, at 0.4 mM in the presence of 1 mM-ATP, and at 3 mM in the presence of 3 mM-2,3-DPG, with and without 1 mM-ATP (Fig. 5). P_i decreased the affinity of the enzyme for both stimulators, thereby increasing sigmoidicity; at 1 mM- P_i , half-maximal stimulation by 2,3-DPG and by ATP were obtained at respectively 5- and 2-fold higher concentrations of these effectors than without P_i (results not shown). When 2,3-DPG, ATP and P_i were added at the concentrations of their unbound form normally prevailing in the erythrocyte, namely 3, 1 and 1 mM respectively, the effect of both stimulators largely preponderated over that of P_i , even at low substrate concentration (results not shown).

5'-Nucleotidase activities in outdated as compared with fresh erythrocytes

When a haemolysate prepared from outdated blood was applied to a DEAE-Trisacryl column and eluted with a linear gradient of NaCl, the second peak of 5'-nucleotidase activity, measured as described in Fig. 1, was much decreased (Fig. 6a). The knowledge that 2,3-DPG, which co-eluted with peak II in the chromatographic system, has completely disappeared in outdated blood prompted its inclusion in the assay of the fractions. As expected from the results obtained with fresh blood, the 5'-nucleotidase activities of peak I were not modified by the addition of 3 mM-2,3-DPG; those of peak II were stimulated 7–8-fold. Unexpectedly, however, a third, intermediate, peak was revealed by this procedure. Eluted at 160 mM-NaCl, it represented about 60 % of the total 5'-nucleotidase activity and 85 % of that stimulated

Fig. 7. Influence of preincubation with 2,3-DPG on the elution profile from the DEAE-Trisacryl column of a haemolysate from outdated blood

Columns were prepared and eluted, and activities measured, as described in Fig. 6. Fraction volume was 3 ml. Panel (a) shows the profile recorded without addition of 2,3-DPG, and panel (b) that obtained after prior overnight incubation of the same haemolysate at 4 °C with 2 mM-2,3-DPG.

by 2,3-DPG. Because of this observation, the activities of the fractions obtained by DEAE-Trisacryl chromatography of a haemolysate from fresh blood were similarly measured in the presence of 3 mM-2,3-DPG (Fig. 6b). Under these conditions the intermediate peak was also revealed, but represented only 29% of the total 5'-nucleotidase activity and 38% of the activity stimulated by 2,3-DPG.

Further investigations of the intermediate peak revealed that it was eluted before 2,3-DPG from the DEAE-Trisacryl column and contained approx. 0.5 mM- P_i . After filtration on Sephadex G-25 or further purification on Blue Sepharose, the following kinetic properties of the 5'-nucleotidase activity of the intermediate peak were found to be closely similar to those of the enzyme purified from peak II: optimum pH, K_m for IMP in the absence and in the presence of 2,3-DPG, half-maximal stimulation by 2,3-DPG and ATP, half-maximal inhibition by P_i . M_r values of the two peaks, estimated by filtration on Sephacryl S-300, were also

similar and not modified by inclusion of 2 mM-2,3-DPG in the elution buffer. It was therefore hypothesized that the retention on DEAE-Trisacryl of the purine 5'-nucleotidase stimulated by 2,3-DPG was influenced by the presence of the stimulator in the haemolysate. To test this hypothesis, 2 mM-2,3-DPG was added before DEAE-Trisacryl chromatography to a haemolysate prepared from outdated blood. The 5'-nucleotidase activity of the intermediate peak, which represented 80% of the total 2,3-DPG-stimulated 5'-nucleotidase activity without addition of 2,3-DPG (Fig. 7a), decreased to 35% as a result of this addition, and the activity of peak II increased commensurately (Fig. 7b). This conversion of the intermediate peak into peak II could also be accomplished by adding 2,3-DPG to the intermediate peak after its elution from the DEAE-Trisacryl column (results not shown). Conversion of peak II into the intermediate peak could also be obtained by freeing peak II of 2,3-DPG by gel filtration (results not shown).

DISCUSSION

As shown in this work, human erythrocytes contain a purine 5'-nucleotidase, which can be separated from other 5'-nucleotide-dephosphorylating activities. The enzyme hydrolyses preferentially, although not exclusively, IMP, GMP and their deoxy counterparts, and displays much less activity on AMP. However, the physiological role of the enzyme is not immediately apparent, since these nucleotides can also be degraded by other 5'-nucleotidases that can be identified in the haemolysate. The properties of the purine 5'-nucleotidase and its physiological function will be discussed separately.

Properties of the erythrocyte purine 5'-nucleotidase

The kinetic characteristics of the purine 5'-nucleotidase isolated from human erythrocytes are in many respects similar to those of the cytosolic 5'-nucleotidases of other tissues. This includes an absolute dependency on Mg^{2+} , a pH optimum around 6.3–6.5 and a higher affinity for inosine and guanosine 5'-nucleotides than for AMP and other nucleoside monophosphates [1–5,26].

Similarly to other cytosolic 5'-nucleotidases, the purine 5'-nucleotidase was stimulated by nucleoside triphosphates, mainly ATP, dATP and GTP, and inhibited by P_i [3–5]. Most noteworthy, the erythrocyte purine 5'-nucleotidase was strongly stimulated by concentrations of 2,3-DPG far below the physiological range. The bisphosphate ester was the most potent stimulator of the enzyme and over-rode the effects of other effectors. Indeed, at the concentration of free 2,3-DPG prevailing in the human erythrocyte under physiological conditions (approx. 3 mM), the enzyme was not only maximally stimulated, but also nearly insensitive to variations of ATP and P_i in the physiological range. 2,3-DPG is known to inhibit several erythrocytic enzymes (reviewed in ref. [27]), among them AMP deaminase [28–30]. The erythrocyte purine 5'-nucleotidase is thus the only enzyme which has been reported hitherto to be stimulated by 2,3-DPG. The apparent M_r of the 2,3-DPG-stimulated purine 5'-nucleotidase of human erythrocytes, namely 250 000, is close to that of the cytosolic 5'-nucleotidase of chicken [26] and rat [31] liver.

As apparent from the different chromatographic behaviour of the purine 5'-nucleotidase when isolated from fresh as compared with outdated erythrocytes (Fig. 6), and from the effects of the addition of 2,3-DPG on this behaviour (Fig. 7), the stimulator also influences the binding of the enzyme to DEAE-Trisacryl. This suggests that the erythrocyte purine 5'-nucleotidase can exist under two different ionic forms, of which the ratio varies in a reversible way according to the concentration of 2,3-DPG, or perhaps of other metabolites. The observation that both forms have similar kinetic characteristics and close M_r values rules out an age-related spontaneous dissociation of the enzyme into smaller components, as has been described for human erythrocyte phosphoribosyl pyrophosphate synthetase (EC 2.7.6.1) [32].

Physiological function of the erythrocyte purine 5'-nucleotidase

Although the purine 5'-nucleotidase is also capable of dephosphorylating purine 5'-deoxyribonucleotides and pyrimidine 5'-ribo- and deoxyribo-nucleotides, two observations indicate that it is unlikely that it plays a significant role in the physiological degradation of the

last three classes of nucleotides. Firstly, the major fractions of the dIMP-, CMP- and dUMP-dephosphorylating activities of the haemolysate, measured at a substrate concentration in the physiological range and at physiological pH, were eluted in peak I from the DEAE-Trisacryl column (Fig. 1). The purine 5'-nucleotidase, although active on high concentrations of pyrimidine 5'-ribo- and deoxyribo-nucleotides (Table 4), is nearly inactive on physiological concentrations of the same compounds, in the absence as well as in the presence of 2,3-DPG. Substantially higher activities were measured with physiological concentrations of dIMP and dGMP. Nevertheless, as evidenced in Fig. 1, even the 2,3-DPG-stimulated activity of peak II with dIMP (owing to the co-elution of 2,3-DPG from the haemolysate in the same fractions) represents only a few per cent of the total activity toward this substrate. Secondly, the stimulatory effect of 2,3-DPG on the 5'-nucleotidase activities in the dialysed haemolysates, which can be considered indicative of the contribution of the 2,3-DPG-stimulated purine 5'-nucleotidase to the overall dephosphorylation process, was clearly evident with IMP and GMP, absent with CMP and dUMP, and not significant with dIMP (Table 1).

The analysis of the dephosphorylating activities of peak I indicates that these fractions contain the specific pyrimidine 5'-nucleotidase described by Paglia & Valentine [10]. This is evidenced by its optimal activity at pH 7.5 (see also Table 2) and apparent M_r close to 28 000 [33]. Peak I presumably also contains the deoxyribonucleotidase with broad specificity and pH optimum around 6.2 (see also Table 2) described by Paglia *et al.* [12]. According to our results, the latter enzyme should have a slightly heavier M_r than the pyrimidine 5'-nucleotidase, namely 45 000.

That the dephosphorylation of erythrocytic AMP, induced, e.g., by deoxyglucose [6] or by glucose deprivation [9], proceeds by way of the purine 5'-nucleotidase is also improbable. Indeed, unlike the IMP-dephosphorylating activity of the haemolysate, that for AMP was eluted predominantly in peak I from the DEAE-Trisacryl column (Fig. 1). This finding, together with the barely detectable activity of the purine 5'-nucleotidase toward physiological concentrations of AMP, and the minimal stimulatory effect of 2,3-DPG on the hydrolysis of AMP in the haemolysate (Table 1), suggests involvement of a 5'-nucleotidase present in peak I. Comparison of the elution profile of the AMP-dephosphorylating activity of peak I with that of CMP (Fig. 1), and the similar M_r values of both enzymic activities, are indicative of degradation of both substrates by the pyrimidine 5'-nucleotidase. However, the different pH optimum of both activities (Table 2) suggests involvement of still another enzyme. Further unravelling of the various 5'-nucleotidase activities present in peak I will be required to resolve this question.

Comparison of the IMP- and GMP-dephosphorylating activities of the purine 5'-nucleotidase purified from peak II with those of peak I leads to the conclusion that these substrates are most likely degraded by the 2,3-DPG-stimulated purine 5'-nucleotidase. The second step of the catabolism of the erythrocyte adenine nucleotides, either under physiological conditions [9] or induced by inhibitors of glycolysis [8] or by alkalization [9], namely the dephosphorylation of IMP generated by deamination of AMP, should thus be mainly catalysed by the purine

5'-nucleotidase purified in this work. This conclusion, as well as the importance of the stimulation of the enzyme by 2,3-DPG, are further substantiated by the higher efficiency of the dephosphorylation of IMP in fresh erythrocytes as compared with outdated cells [8].

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REFERENCES

- Itoh, R., Mitsui, A. & Tsushima, K. (1968) *J. Biochem. (Tokyo)* **63**, 165–169
- Fritzson, P. (1969) *Biochim. Biophys. Acta* **178**, 534–541
- Van den Berghe, G., Van Pottelsberghe, C. & Hers, H. G. (1977) *Biochem. J.* **162**, 611–616
- Itoh, R. (1981) *Biochim. Biophys. Acta* **657**, 402–410
- Carson, D. A. & Wasson, D. B. (1982) *Cancer Res.* **42**, 4321–4324
- Henderson, J. F., Zombor, G., Burrige, P. W., Barankiewicz, G. & Smith, C. M. (1979) *Can. J. Biochem.* **57**, 873–878
- Mills, G. C., Goldblum, R. M. & Schmalstieg, F. C. (1981) *Life Sci.* **29**, 1811–1820
- Plagemann, P. G. W., Wohlhueter, R. M. & Kraupp, M. (1985) *Biochim. Biophys. Acta* **817**, 51–60
- Bontemps, F., Van den Berghe, G. & Hers, H. G. (1986) *J. Clin. Invest.* **77**, 824–830
- Paglia, D. E. & Valentine, W. N. (1975) *J. Biol. Chem.* **250**, 7973–7979
- Swallow, D. M., Aziz, I., Hopkinson, D. A. & Miwa, S. (1983) *Ann. Hum. Genet.* **47**, 19–23
- Paglia, D. E., Valentine, W. N. & Brockway, R. A. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 588–592
- Paglia, D. E., Valentine, W. N., Nakatani, M. & Brockway, R. A. (1986) *Blood* **67**, 988–992
- Bontemps, F., Van den Berghe, G. & Hers, H. G. (1985) *Pediatr. Res.* **19**, 747 (abstr.)
- Bontemps, F., Van den Berghe, G. & Hers, H. G. (1986) *Adv. Exp. Med. Biol.* **195(B)**, 283–290
- Crabtree, G. W. & Henderson, J. F. (1971) *Cancer Res.* **31**, 985–991
- Agarwal, R. P. & Parks, R. E., Jr. (1977) *Biochem. Pharmacol.* **26**, 663–666
- Van den Berghe, G., Bontemps, F. & Hers, H. G. (1980) *Biochem. J.* **188**, 913–920
- Itaya, K. & Ui, M. (1966) *Clin. Chim. Acta* **14**, 361–366
- Adam, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed), 1st edn., pp. 573–577, Academic Press, London and New York
- Vincent, M. F., Van den Berghe, G. & Hers, H. G. (1984) *Biochem. J.* **222**, 145–155
- Beutler, E., West, C. & Blume, K. G. (1976) *J. Lab. Clin. Med.* **88**, 328–333
- Michal, G. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1492–1495, Academic Press, London
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Tsuboi, K. K. & Hudson, P. B. (1953) *Arch. Biochem. Biophys.* **43**, 339–357
- Naito, Y. & Tsushima, K. (1976) *Biochim. Biophys. Acta* **438**, 159–168
- Chiba, H. & Sasaki, R. (1978) *Curr. Top. Cell. Regul.* **14**, 75–116
- Askari, A. & Rao, S. N. (1968) *Biochim. Biophys. Acta* **151**, 198–203
- Lian, C. Y. & Harkness, D. R. (1974) *Biochim. Biophys. Acta* **341**, 27–40
- Yun, S. L. & Suelter, C. H. (1978) *J. Biol. Chem.* **253**, 404–408
- Itoh, R. (1982) *Biochim. Biophys. Acta* **716**, 110–113
- Yip, L. C., Roome, S. & Balis, M. E. (1978) *Biochemistry* **17**, 3286–3291
- Torrance, J. D., Whittaker, D. & Beutler, E. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3701–3704

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